DISSECTION OF THE MOLECULAR INTERPLAY BETWEEN THE MOSQUITO INNATE IMMUNE SYSTEM, GUT MICROBIOME, AND PATHOGENS

by

April Melinda Clayton

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ABSTRACT

Malaria, caused by the *Plasmodium* parasite, affects approximately 3 billion people worldwide each year. The major vector for *P. falciparum* in sub-Saharan Africa is the female *Anopheles gambiae* mosquito. Given the lack of an effective vaccine against malaria and the increased resistance of this parasite to the current arsenal of drugs and of *Anopheles* mosquitoes to insecticides, the development of novel control strategies is crucial to reducing malaria transmission. Studies exploring the mosquito's innate immune defense against *Plasmodium* as well as studies detailing the importance of the midgut microbiota in vector competence to pathogens may contribute towards the development of effective control strategies. In this thesis work, we have:

1. Conducted RNAi-based reverse genetic assays and high-throughput gene expression analysis to study the implication of the mosquito immune pathways in the response against the human malaria parasite *Plasmodium falciparum*. Additional infection and functional assays showed that the Imd immune pathway factor Caudal is an effective regulator of the anti-*Plasmodium* defense response in the *Anopheles gambiae* mosquito.

2. Studied the cross-colonization capacities between the midgut microbiota and the phylogenetically distinct mosquito species, *Aedes aegypti* and *Anopheles gambiae*. Investigating such interactions may provide insight in how to implement microbiota-based control strategies of different vector-borne diseases. Some bacterial isolates demonstrated the ability to cross-colonize the two mosquito hosts. However, some isolates demonstrated co-species

adaptation rather than cross-colonization capacity.

This thesis work contributes to a better understanding of the mosquito immune system to the malaria parasite and microbiota-mosquito interactions.

Keywords: Imd pathway, innate immunity, malaria, mosquito, *Aedes*, *Anopheles*, *Plasmodium falciparum*, microbiota.

Thesis Advisor: Dr. George Dimopoulos

Thesis Advisory Committee:

Dr. Craig Montell (BCMB Co-advisor)

Dr. Marcelo Jacobs-Lorena

Dr. Erika Matunis

Ph.D. DISSERTATION REFEREES FOR APRIL MELINDA CLAYTON

- George Dimopoulos, PhD, MBA, Professor, Johns Hopkins Bloomberg School of Public Health (faculty sponsor)
- Marcelo Jacobs-Lorena, PhD, Professor, Johns Hopkins Bloomberg School of Public Health (reader)

PREFACE

To my parents, brother, and sisters, whose endless love, unconditional support, sacrifice, and advice allowed me to continue doing what I love: research; to all my friends, who have a special place in my heart; and to my Lord and Savior for sustaining me.

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CHAPTER 1
INTRODUCTION

Portions of this work has been submitted and accepted for publication: **Clayton, AM**, Dong, Y, and Dimopoulos, G. **(2013)**. Scientific Review: *Anopheles* innate immune defenses against *Plasmodium* infection. **Submitted and accepted to the Journal of Innate Immunity**.

Malaria, caused by the *Plasmodium* parasite, affects approximately 3 billion people worldwide each year. The major vector for *P. falciparum* in sub-Saharan Africa is the female *Anopheles gambiae* mosquito. Given the lack of an effective vaccine against *Plasmodium* and the increased resistance of this parasite to the current arsenal of drugs and of *Anopheles* mosquitoes to insecticides, the development of novel control strategies is crucial to reducing malaria transmission [1]. Studies exploring the mosquito's innate immune defense against *Plasmodium* as well as the mosquito midgut microbiota may contribute towards the development of such preventive and control strategies. In this chapter, we will discuss recent findings from studies investigating anti-*Plasmodium* defenses in the mosquito, with a specific focus on those involved in parasite elimination in the midgut. Additionally, this chapter specifies the aims and questions that led to the development of this doctoral research thesis.

Plasmodium **infection of the** ***Anopheles*** **mosquito**

Plasmodium transmission requires that the parasite complete an intricate replicative cycle in the mosquito that involves transitions through several developmental stages and interactions with the mosquito's midgut and salivary gland tissues as well as the hemocoel. This journey takes approximately 2-3 weeks (the time varies for different *Plasmodium* species and strains) and begins when the female mosquito ingests a blood meal infected with *Plasmodium* gametocytes. The male and female gametocytes develop into male microgametes and female macrogametes, respectively, in the midgut lumen. Fertilization of the gametes results in the formation of zygotes. The zygotes then transform into motile ookinetes that invade and migrate across the midgut epithelium,

roughly 18-36 hours after the ingestion of an infected blood meal. The route of ookinete invasion across the midgut epithelium as well as the cellular responses of the midgut epithelium to ookinete invasion are still topics of controversy despite numerous studies involving diverse *Plasmodium*-mosquito combinations [2-4]. However, these responses generally involve apoptosis and an extrusion of ookinete-invaded midgut epithelial cells into the midgut lumen [5-12]. Once the diploid ookinete has reached the basal side of the midgut epithelium, it transforms into an oocyst and undergoes several rounds of replication by means of sporogony. Approximately 10-12 days after the blood meal, each oocyst contains thousands of haploid sporozoites, which are then released into the mosquito hemocoel at about 14 days after the blood meal and migrate through the mosquito hemolymph in order to invade the salivary glands. During the next blood meal, these *Plasmodium* sporozoites are injected with the saliva into the human (or another vertebrate) host, thereby completing the sexual cycle of *Plasmodium* within the mosquito vector [13,14].

Mosquito immune signaling pathways in the defense against *Plasmodium*

In order to continue its cycle of transmission and eventual infection of the human host, the malaria parasite engages in a series of complex interactions with the mosquito vector. Parasite numbers are limited by several major bottlenecks that occur in the mosquito such as when the ookinete traverses the midgut epithelium prior to the development of the oocysts on the basal side and during the migration of sporozoites to the salivary glands. [15-19]. The mosquito's innate immune system has been shown to play a key role in killing parasites and thereby affecting parasite development [20,21].

The two major arms of the insect innate immune response are: 1) a humoral response involving, for examples, a complement-like system and the transcriptional upregulation of small cationic antimicrobial peptides (AMPs) and other immune effectors and 2) a cell-mediated response that includes phagocytosis and/or melanization. Other defenses include oxidative and nitric oxide-mediated killing mechanisms.

As earlier mentioned, the innate immune system of *Anopheles,* the mosquito's main line of defense against parasites, fungi, bacteria, and viruses, is engaged at multiple stages of *Plasmodium* infection [13,22-24]. Three major signaling pathways contribute to anti-*Plasmodium* defense: the Toll, the immune deficiency (Imd), and the Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathways (Fig. 1.1). Considerable insight into these innate immune pathways has been gathered from studies conducted in *Drosophila* [24,25].

The Toll and Imd pathways

The mosquito's anti-*Plasmodium* and antibacterial defenses are largely controlled by the Toll and Imd NF-kappaB immune signaling pathways (Fig. 1.1). The Toll pathway is primarily elicited by Gram-positive (G+) bacteria, fungi, and *Plasmodium*. Studies have also implicated this pathway in the defense against viruses [26]. The Imd pathway is elicited by Gram-negative (G-) and G+ bacteria and *Plasmodium* [22,27].

Infection-responsive activation of the Toll and Imd pathways via the recognition of pathogen-associated molecular patterns (PAMPs) ultimately leads to the nuclear translocation of the NF-kappaB transcription factors Rel1 and Rel2, respectively (Fig. 1.1). These transcription factors are negatively regulated in the cytoplasm by Cactus and Caspar, respectively. Activation of the Toll and Imd pathways allows the Rel factors to

enter the nucleus and transcriptionally activate immune effector genes such as antimicrobial peptides (AMPs) and other factors. The four main classes of AMPs are defensins, cecropins, attacin, and gambicin. These AMPs act against G- and G+ bacteria, yeast, fungi, and *Plasmodium*. Actually, gambicin was among the first anti-*Plasmodium* factors identified [28]. Additionally, it has been demonstrated that both the Rel1 and Rel2 transcription factors can induce the expression of the AMP genes *Cecropin 1*, *Defensin 1*, and *Gambicin 1* [29].

The Imd pathway-controlled transcription factor *Rel*2 gene produces a full-length form (*Rel*2-F) that includes the carboxyl-terminal ankyrin (ANK) and death domains as well as a shorter form (*Rel*2-S) lacking such domains due to alternative splicing. The *Rel2*-S form is constitutively translocated to the nucleus, where it regulates the transcription of immune genes [27] (Fig. 1.1).

While the Toll pathway has been shown to be more effective in the defense against the rodent *P. berghei* parasite, the Imd pathway has emerged as the most effective pathway in the defense against the human malaria parasite *P. falciparum* [30-33]. Specifically, activation of the Imd pathway by the gene silencing, via RNAi, of *Caspar* (a suppressor of the Imd pathway) results in an Imd pathway-mediated immune defense that confers almost complete refractoriness to *P. falciparum* in three major *Anopheles* malaria vector species: *An. gambiae*, *An. stephensi*, and *An. albimanus*. In contrast, activation of the Toll pathway by the silencing of *Cactus* (a suppressor of the Toll pathway) results in a significantly greater resistance to infection with the rodent malaria parasite *P. berghei* [31]. These two *Plasmodium* species elicit diverse innate immune responses at the gene transcript level [34]. A diverse repertoire of anti-*Plasmodium* immune effectors regulated

by the Imd pathway, including APL1, TEP1, LRRD7 (APL2), FBN9, and LRIM1 have been identified and studied with regard to their antiparasitic action and will be discussed later [27,33-39]. It has also demonstrated and characterized the potency of the Imd pathway in anti-*Plasmodium* defense through the use of genetically modified immune-enhanced *Anopheles* mosquitoes that express blood meal-inducible *Rel*2 in both the midgut and fat body tissues [40]. The transient activation of this transgene resulted in almost complete resistance to the human malaria parasite at a negligible fitness cost, prompting further investigation of this system as an innovative malaria control strategy [40].

The potency of the Imd pathway in the anti-*P. falciparum* response has warranted further molecular dissection in light of recent studies [31,32,40]. There are, for example, only a few studies detailing the regulation of Rel2 once it has been translocated to the nucleus. Recent work has demonstrated that the transcription factor Caudal (Cad) is an antagonist of Rel2 (see Fig. 1.1) and also a negative regulator of the Imd pathway's anti-*P. falciparum* defense in the *Anopheles* mosquito [41]. Cad was previously identified as a negative regulator of the Imd pathway in adult *Drosophila* [42].

RNAi-mediated silencing of *Cad* specifically compromised *P. falciparum* development in the gut tissue, suppressed the midgut microflora, and enhanced resistance to systemic bacterial infections, most likely by causing an increased transcriptional abundance of AMPs and other effector genes. Interestingly, *Cad* gene silencing resulted in increased longevity in the female adult mosquitoes, but the silencing of *Cad* impaired the mosquito's fecundity and fertility [41], indicating that Cad may display functional diversity in terms of immunity, development, and perhaps other processes.

Another study has implicated the transcriptional mediators Kohtalo (Kto) and Skuld (Skd) as participants in the regulation of the Imd pathway's anti-*P. falciparum* defense in *An. gambiae*. Depletion of the *Kto* and *Skd* genes by RNAi in the mosquito resulted in an increased susceptibility to bacterial and human malaria parasite infection, but not to infection with the rodent malaria parasite *P. berghei* [43].

The JAK-STAT pathway

Little is known about the role of JAK-STAT in insects; however, in *Drosophila*, this pathway is involved in a variety of developmental processes. It has also been implicated in antibacterial and antiviral defense in *Drosophila* and the *Aedes* mosquito [22,44,45]. Recent studies in *Anopheles* have also linked this pathway to anti-*Plasmodium* defense [46-48]. In *Drosophila,* this signaling pathway is initiated by the binding of the cytokine ligand Unpaired (UPD) to the transmembrane receptor Domeless (DOME), leading to the phosphorylation of DOME by the JAK tyrosine kinase Hopscotch (HOP) (Fig. 1.1). Phosphorylation of DOME recruits a STAT, which is then phosphorylated, dimerized, and translocated to the nucleus, where it transcriptionally upregulates immune effector genes. This pathway is tightly regulated by proteins such as the suppressor of cytokine signaling (SOCS) and the protein inhibitor of activated STAT (PIAS). SOCS is transcriptionally activated by this pathway as part of a negative feedback loop that regulates STAT signaling by preventing STAT phosphorylation. PIAS inhibits signaling by binding to STAT proteins and targeting them for degradation [49,50]. There are two STAT genes in *An. gambiae* (*STAT1*/*AgSTAT-B* and *STAT2*/*AgSTAT-A*), and only one in *Drosophila* (*Stat92E*); a one-to-one orthology relationship exists for JAK and DOME in

these two species [24,51] (Fig. 1.1).

The JAK-STAT pathway mediates immunity against the malaria parasite through both STAT genes, *AgSTAT-A* and *AgSTAT-B*. *AgSTAT-A* is an ancestral gene regulated at the mRNA level by the *AgSTAT-B* gene. *Ag*STAT-A has recently been shown to mediate the transcriptional activation of nitric oxide synthase (NOS), which is induced in response to *Plasmodium* infection and leads to high levels of reactive nitric oxide (NO), thereby diminishing parasite development. *Ag*STAT-A also activates the transcription of SOCS. Silencing of *AgSTAT-A* increases mature oocyst development in *P. berghei* and *P. falciparum*-infected mosquitoes [46]. These findings suggest that the JAK-STAT pathway regulates NOS expression and induces immunity to the later oocyst stages of *Plasmodium* in the *An. gambiae* midgut. However, Bahia and colleagues have recently shown that the JAK-STAT pathway controls the early stages of infection with *P. vivax*, another virulent form of human malaria, in the Brazilian malaria vector *Anopheles aquasalis* [47].

While the Toll, Imd, and JAK-STAT are the best characterized pathways, however other pathways have also been shown to play key roles in antiplasmodial immunity such as the insulin/insulin growth factor-1 (IGF-1) signaling (IIS) pathway. The activation of the IIS pathway increases susceptibility to *P. falciparum* in *An. stephensi* and may even alter NF-kappaB-dependent immunity [52-54].

Anopheles molecular immune responses to *Plasmodium* infection

The past 20 years have witnessed great progress in understanding the mosquito's immune system, and a variety of putative immune genes/effectors have been implicated

in the defense against *Plasmodium* (reviewed in [22,51]). In particular, ookinete invasion of the midgut epithelium by different *Plasmodium* species results in the elicitation of both common and diverse molecular responses [7,34,55]. These global transcriptomic analyses have identified a plethora of genes that were later shown to represent key players in anti-*Plasmodium* defense.

One of the first anti-*Plasmodium* factors studied was the hemocyte-specific thioester complement-like protein TEP1, which binds to, and mediates killing of *P. berghei* ookinetes. TEP1 is upregulated 24 hours after ingestion of either *P. berghei- or P. falciparum*- infected blood and plays a role in the defense against both *Plasmodium spp.* [31,34,40,56]. Two leucine-rich repeat (LRR) proteins, LRIM1 and APL1C, are factors that function with TEP1 to regulate *Plasmodium* loads in the mosquito. Together, these three factors establish a complement-like pathway that is pivotal for antiplasmodial defense [39]. This defense mechanism is discussed in greater detail later in this review.

In *An. gambiae,* the superfamily of LRR domain-containing proteins is a gene family that encodes secreted, membrane-bound or cytoplasmic proteins with diverse functions; LRR immune proteins (LRIM) are members within this superfamily and have been shown to be prominent players in the antiplasmodial response[36,57]. LRIM1 is upregulated in *An. gambiae* after infection with *Plasmodium* [34]. Additionally, LRIM1 is a key antagonist of *P. berghei* and causes a substantial majority of the ookinetes to be killed while traversing the midgut, before oocyst formation [21,36]. Two other LRIM family members, *Anopheles Plasmodium*-responsive leucine-rich repeat 1 (APL1) and LRRD7, have also been shown to be involved in the defense of both *P. falciparum* and *P. berghei* development in the mosquito [21,34,35,58].

Interestingly, a recent study has shown that the *APL1* locus encodes three genes, *APL1A*, *APL1B*, and *APL1C*, which share more than 50% identity at the amino acid level [35]. This locus lies within a quantitative trait locus (QTL) that confers vector resistance to *P. falciparum* in wild mosquito populations in Africa [17,58-60]. Rottschaefer and colleagues recently examined the molecular genetic variation in the *APL1* locus in diverse West African collections of *An. gambiae*, and they found that the *APL1* locus is extremely polymorphic [37]. Within these paralogs, the *APL1A* gene was thought to be involved in the defense against *P. falciparum* through the Imd pathway. In contrast, the same study indicated that the gene *APL1C* protects the mosquito against the rodent malaria parasites *P. berghei* and *P. yoelii* only through the Toll signaling pathway [33]. Another study of the *APL1* genes showed that they behaved differently than reported by Mitri and colleagues [32]. While the role for *APL1* genes in limiting *P. falciparum* infection was confirmed, a significant role for the *APL1A* gene in the anti-*P. falciparum* immune response was not apparent. However, silencing of *APL1B* and *APL1C* had a significant impact on *P. falciparum* infection. As earlier stated, the *APL1* gene family has exhibited a complex sequence evolution, including an exceptionally high degree of polymorphism [37]. Therefore, although the latter study confirms a role for *APL1* gene family members during *P. falciparum* infection, the differences between the two studies may be explained by different versions of *APL1* sequences in the used mosquito strains, or the fact that different *P. falciparum* parasite genotype resulting in different infection intensities were used in the two studies.

Another class of *Plasmodium* effectors is the c-type lectins (CTL). Two members of this family, CTL4 and CTLMA2, are present in the hemolymph of *An. gambiae* and

their transcripts are both upregulated 24 hours after blood-feeding on *P. berghei* infected mice [21]. Interestingly, these two CTLs can protect the rodent *Plasmodium* ookinetes from destruction [21]. CTL4 is also induced by *P. falciparum*-infected blood with non-invading ookinetes, while CTLGA3 is induced by invading *P. falciparum* ookinetes [34]. CTL4 and CTLMA2 are soluble proteins that are secreted in the hemolymph in the form of a disulfide-linked heterodimeric complex (similar to the LRIM1/APL1C complex which will be discussed later in this review) and protect the mosquito from infection by G- bacteria [61]. This mode of action may provide a link between their role in antibacterial defense and the melanization of *P. berghei*.

Components of the lipid transporting system, such as apolipophorin and apolipoprotein D precursors, also have a significant impact on *Plasmodium* development [34,62,63]. An apolipophorin precursor, RFABG, is induced by *P. berghei* invasion [62], and the transcript level of the apolipoprotein D (APOD) gene is increased upon *P. falciparum* infection [34]. Apolipophorin-III (ApoLp-III) has recently been identified as a player in midgut antiplasmodial defense. ApoLp-III mRNA is strongly expressed in the *Anopheles* midgut upon *P. berghei* infection; in addition, silencing of the *ApoLp-III* gene significantly increases *P. berghei* oocyst levels [63]. Work by Rono and colleagues demonstrated that lipophorin (Lp) reduces the parasite killing efficiency of TEP1; however the absence of Lp increased TEP1's efficiency to bind to *Plasmodium* ookinetes [64].

The fibrinogen-related proteins (FREPs) are a pattern recognition receptor (PRR) family that also exhibits anti-*Plasmodium* activity. The *FREP* gene family is significantly expanded in *An. gambiae*, with 58 members, as compared to 37 members in the mosquito

Aedes aegypti and only 14 in *D. melanogaster* [65-68]. RNAi-mediated gene-silencing assays have indicated that the *FBN8*, *FBN9*, and *FBN39* genes are involved in the anti-*Plasmodium* defense; their involvement is specific, with FBN39 regulating only the mosquito's resistance to the human malaria parasite, and FBN9 and FBN8 being induced in response to both *P. berghei* and *P. falciparum* infection [34,65,69,70].

The G- bacteria-binding proteins (GNBPS) represent another PRR family that is important in antimalarial defense. GNBPB3 and GNBPB4 are only upregulated after challenge with *P. berghei*, and GNBPB1 is induced only by *P. falciparum*-infected blood [34,71]. Also, within the class of PRRs in *An. gambiae* are the splice variants of the *An. gambiae* Down syndrome cell adhesion molecule gene (*AgDscam*), which has been shown to protect mosquitoes against challenge with either *P. berghei* or *P. falciparum* [72,73]. The *AgDscam* gene has been identified as a hypervariable PRR with the potential to generate 31,000 alternative splice forms that are responsible for different pathogen interactions and specificities. Specifically, the Imd and Toll pathways mediate *Ag*Dscam-mediated species-specific defenses against *Plasmodium* and bacteria by regulating the alternative splicing of this gene [73]. The Imd pathway-controlled immune-responsive splicing factors Caper and IRSF1 regulate *Ag*Dscam splicing and influence anti-*Plasmodium* defense specificity. Imd pathway activation was also shown to enhance the association of *Ag*Dscam with *P. falciparum* ookinetes in the mosquito midgut epithelium [73].

Anti-*Plasmodium* defense mechanisms

The "Time Bomb" Theory

Midgut invasion by the *Plasmodium* ookinete does not leave the mosquito unharmed. According to the "Time Bomb" theory, a model of the cellular and molecular response of the *An. stephensi* midgut epithelium to *P. berghei* ookinete invasion, invading ookinetes inflict irreversible damage on the midgut epithelial cells as the parasite moves in order to reach the basal lamina, where it differentiates into an oocyst [8,74]. The invaded cells upregulate NOS expression, have fewer microvilli, undergo DNA fragmentation, and possess abnormally shaped nuclei and a remodeled actin cytoskeleton. In addition to causing cellular damage and eliciting molecular responses, the ookinetes also secrete the Pbs21 surface protein and PbSub2 protease, which may help facilitate the motility of the ookinete as it glides across the epithelium. The defense response of elevated NOS expression (and consequent NO generation) and the initiation of cell death and protrusion create a ticking time bomb and an altogether hostile environment for the traversing ookinete. Thus, the ookinete must move quickly from the damaged cells in order to continue its development in the mosquito midgut. While the majority of studies have been conducted in *An. stephensi*, NOS expression is known to be elevated in *An. gambiae* after *P. berghei* infection [20,75]. Biochemical studies in *An. gambiae* reveal nitration in *Plasmodium*-invaded midgut cells to occur as a two-step process in which the induction of NOS expression is followed by peroxidase activity [76,77]. Recent work identified heme peroxidase 2 (HPX2) and NADPH oxidase 5 (NOX5) as mediators of nitration in the *An. gambiae* midgut epithelium and demonstrated that epithelial nitration and TEP1-mediated lysis work sequentially to target *Plasmodium* ookinetes. The authors propose that nitration of ookinetes in the midgut promotes the subsequent activation of the mosquito complement system [78].

Work conducted by Shiao and colleagues proposes a wound-healing response mechanism to dead or dying ookinetes that has been argued to be in conflict with the "Time Bomb" theory [79]. In this study, the authors claim that while the majority of *P. berghei* ookinetes are killed in the extracellular space in *An. gambiae*, dead or dying ookinetes are surrounded by a polymerized actin zone formed at the basal layer of adjacent midgut epithelial cells. The formation of this zone is strongly linked to the activation of the melanization response (which is discussed later in this review). Furthermore, the study identified two factors controlling the formation of the actin zone and subsequent activation of melanization: the transmembrane receptor frizzled-2 (Fz2) and the guanosine triphosphate-binding protein cell division cycle 42 (Cdc42). Discussed later in this review, RNAi-mediated silencing of these two factors did not affect ookinete survival. Collectively, these results suggest a separation of parasite killing from subsequent reactions manifested by actin zone formation (in this case, the activation of melanization) [79].

The mosquito complement system

The complement cascade in the *Anopheles* hemolymph has emerged as a key antiplasmodial defense mechanism. As previously discussed, the mosquito complement C3-like protein TEP1 binds to the surface of midgut-invading ookinetes and marks them for killing [38]. TEP1 circulates in the mosquito hemolymph as a full-length protein and a processed form, $TEP1_{cut}$. Recent studies have independently revealed that the *An. gambiae* LRR proteins LRIM1 and APL1C are circulated in the hemolymph as a disulfide-linked heterodimer [36,39]. This complex interacts with and stabilizes $TEP1_{cut}$

and is required for TEP1 accumulation on the ookinete surface. These results reveal that the LRIM1/APL1C/TEP1$_{cut}$ complex functions as a complement-like system for parasite killing. They also indicate a potential role for the LRIM1/APL1C complex in binding multiple targets, as mammalian multi-subunit receptors have similarly been shown to robustly activate the complement pathway. The conformational changes in the LRIM1/APL1C complex can then facilitate the recruitment of additional cascade components such as TEP1-activated proteases. A recent paper has suggested that a cleaved form of TEP1 can act as a convertase for the activation of other TEP1 molecules and that the LRIM1/APL1C complex may regulate the formation of this TEP1 convertase [80]. Future in-depth studies of this complex will provide more detailed insight into complement activation and its role in *Plasmodium* killing.

Hemocyte-mediated defenses

Insect blood cells known as hemocytes (which are macrophage-like) play a key role in the mosquito innate immune response against pathogens and exist in the insect's open circulatory system. These cells function in defense against pathogens either directly through phagocytosis or indirectly through secretion of effectors such as AMPs, complement-like proteins, and effectors of the melanization response [81]. The hemolymph of the *An. gambiae* adult female contains three hemocyte sub-types: granulocytes, oenocytoids, and prohemocytes. These types can be distinguished from one another by morphological and functional markers. Granulocytes function in phagocytosis, oenocytoids play a role in melanization, and the prohemocytes are hypothesized to serve as hematopoietic progenitors [82]. Work by King and Hillyer has identified a novel type

of hemocytes, known as periostial hemocytes, which surround the heart in order to phagocytose bacterial and *Plasmodium* pathogens as they flow in the hemolymph, highlighting the interaction among the mosquito innate immune and circulatory systems [83].

Transcriptomic profiles of adult female *An. gambiae* hemocytes following bacteria and *Plasmodium* infection revealed pathogen-specific signatures of gene regulation and expression. Particularly, 4,047 genes were expressed, with 959 genes being differentially expressed following bacteria or *Plasmodium* challenge [84]. In addition to varied transcriptomic profiles, the number of circulating hemocytes in adult mosquitoes change in response to infection as well as age and physiological state [85-88]. It has been speculated that such changes may be due to a release of sessile hemocytes (hemocytes attached to tissues) or differentiation of the prohemocytes [82,86,87,89,90]. A recent study that investigated the *in vivo* distribution of hemocytes in adult *An. gambiae* demonstrated that the increase and proliferation of circulating hemocytes following infection is primarily due to mitosis in the circulating hemocytes rather than the differentiation of a progenitor cell type [91].

Interestingly, the differentiation of hemocytes has been implicated in facilitating innate immune memory in *An. gambiae.* It is much understood that the innate immune system is unable to establish memory in a fashion similar to the adaptive immune system (which is not present in insects). However, memory-like responses, termed immune priming, have been described in insects (as well as other invertebrates) [92-96]. Recent work has demonstrated an immune priming mechanism in mosquitoes in response to

Plasmodium in the presence of their midgut microbiota. Particularly, this memory was shown to be primed by the invasion of the mosquito midgut by ookinetes. Ookinete invasion resulted in a long-lasting increase in granulocytes and enhanced immunity to bacteria. This enhanced antibacterial immunity indirectly reduced *Plasmodium* parasite survival upon reinfection [86].

While the three sub-types of hemocytes are much agreed upon, the number of circulating hemocytes within the adult mosquito is still a source of debate. For example, the authors of the aforementioned study based their conclusions on mosquitoes containing an estimated range of 30,000-50,000 circulating hemocytes [86]. However, other studies have determined that the range of circulating hemocytes in adult mosquitoes and *Drosophila* flies is between 1,000 and 5,000 [82,85,87-89,91,97]. Such discrepancies may provide an impetus to further investigate the basic aspects of hemocyte biology in addition to the methods employed to isolate and count hemocytes.

Melanization in the anti-Plasmodium defense response

Melanization is another innate immune response in the mosquito that the *Plasmodium* parasite may face. This innate immune mechanism has been genetically mapped to three QTL in *An. gambiae,* collectively called the *Plasmodium* encapsulation genes: *Pen1*, *Pen2*, and *Pen3* [98,99]. Melanin formation in the mosquito is a result of the proteolytic activation of prophenoloxidase (PPO) to phenoloxidase (PO), induced by a cascade of CLIP serine proteases. PO then oxidizes tyrosine and 3, 4-dihydroxy phenylalanine (DOPA) to form reactive quinones that produce melanin. When a pathogen invades the mosquito, the mosquito deposits melanin, which then crosslinks proteins and forms a capsule around the parasite (reviewed in [100]). The melanization

process is highly regulated by serine protease inhibitors, or serpins (SRPNs), which block the activation of PO (reviewed in [101]).

Genetically selected refractory (R) and susceptible (S) strains of *An. gambiae* have provided valuable insight into the mosquito's melanization mechanism. The R strain is highly efficient at blocking *Plasmodium* development in the midgut via melanization, when compared to the S strain. We will briefly highlight the use of R and S mosquito strains in providing insight about the roles of CLIP serine proteases in melanization.

Silencing of the *CLIPA8* gene in R and in S mosquitoes in which the anti-*Plasmodium* gene *CTL4* has also been silenced has demonstrated that this CLIP protease is essential for activating the PO cascade and hence necessary for the melanization of *P. berghei* ookinetes [102]. Recent work by Yassine and colleagues has also demonstrated the importance of CLIPA8 in the melanization response against the entomopathogenic fungus *Beauveria bassiana* in *An. gambiae* mosquitoes [103]. CLIPA2, A5, and A7 suppress melanization, with CLIPA2 and CLIPA5 acting synergistically to block ookinete invasion. Two CLIPBs, CLIPB14 and CLIPB15, are also involved in the killing of *Plasmodium* ookinetes and participate in the defense against G- bacteria [104]. CLIPB3, B4, B8, and B17 promote ookinete invasion [101,102], and silencing of the *SRPN2* gene increases melanization and reduces the ability of *P. berghei* ookinetes to invade the midgut epithelium [105]. While depletion of the *SRPN2* gene was shown to negatively affect the ability of the parasite to invade the midgut epithelium and develop into oocysts, gene silencing of *SRPN2* in *An. gambiae* mosquitoes originally from Cameroon was not found to influence the development of field strains of *P. falciparum* [106]. The results of this study suggest that some strains of the parasite are efficient at

evading the mosquito's innate immune system.

One study has recently demonstrated that CLIPB9 acts as a PPO-activating proteinase that is inhibited by SRPN2. It also showed that CLIPB9 and SRPN2 not only interact to form a regulatory unit of melanization but also affect the life span of adult female mosquitoes [107]. Another SRPN, SRPN6, mediates the defense against malaria parasites and bacteria. In particular, *SRPN6* gene expression is induced upon infection with *E. coli* and both rodent and human malaria parasites and is specifically expressed in midgut cells invaded by ookinetes and in surrounding hemocytes. Silencing of *SRPN6* in *An. gambiae* has demonstrated that its role in parasite clearance is to inhibit melanization in order to promote parasite lysis [108]. Additionally, silencing of *SRPN6* also reduces sporozoite numbers in the salivary glands [109]. A recent study has shown that the LPS-induced TNFα transcription factor (LITAF)-like 3 (LL3) in *An. gambiae* is capable of modulating *SRPN6* gene expression to influence its anti-*Plasmodium* response [110].

In addition to the aforementioned melanization effectors, other molecules and anti-*Plasmodium* factors also modulate the mosquito's melanization response. In R females, dead *Plasmodium* ookinetes have been shown to associate with a zone of actin in nearby midgut cells and with melanin deposition on the ookinete surface [79]. As discussed earlier in this review, the genes *frizzled-2* (*Fz2*) and *cell division cycle 42* (*Cdc42*) are required for these two processes of actin polymerization and melanization [79]. However, RNAi-mediated silencing of these two genes does not affect the killing of *Plasmodium* ookinetes; rather, these two factors contribute to the mosquito's wound healing mechanism during *Plasmodium* infection. Additionally, gene silencing of *CTL4* and *CTLMA2* resulted in increased ookinete melanization [21].

TEP1 may also play a role in *Plasmodiu*m melanization, since the RNAi-mediated silencing of *TEP1* renders R females unable to melanize *P. berghei,* thereby making them susceptible to infection [38]. However, silencing TEP1 in S mosquitoes increased the number of developing parasites. The results from this work suggests that TEP1-dependent parasite killing is followed by a TEP1-independent clearance of dead parasites by lysis and/or melanization [38]. TEP1 has also been implicated in the melanization response to fungal infection in *An. gambiae* mosquitoes and Sephadex beads [103,111].

Silencing of the complement-like system genes *LRIM1* and *APL1C* also results in a decrease in melanized *Plasmodium* parasites [36]. Work by Warr and colleagues [111] has also indicated that the silencing of *LRIM1* and *TEP1* (as earlier mentioned) compromises the mosquito's ability to melanize Sephadex beads, whereas silencing of *CTL4* and *CTLMA2* did not affect bead melanization.

Recent work has shown that some strains of *P. falciparum* are able to evade this complement-like system (i.e., TEP1, LRIM1, and APL1C) in *An. gambiae*. In particular, this work demonstrated that the silencing of *TEP1*, *LRIM1*, and *APL1C* in *An. gambiae* prevented the melanization of the Brazilian *P. falciparum* 7G8 line. However, there was no effect on infection intensity when the African *P. falciparum* strain NF54 was used, suggesting this line is able to evade this complement-like system. When *An. gambiae* R mosquitoes were co-infected with 7G8 and another African *P. falciparum* strain, 3D7, mixed infections comprised of both live and encapsulated parasites were produced in the midgut, suggesting that survival is parasite-specific in nature [112]. Silencing of *Rel2* and *PGRP-LC* led to melanization of *Plasmodium* in the mosquito midgut, suggesting that the

Imd pathway is a negative regulator of the melanization response in the mosquito [27,30,113].

Mosquito – bacteria interactions

The mosquito midgut microbiota

The presence of bacteria in the midgut (the midgut microbiota) stimulate a basal innate immune activity consisting of the induction of AMPs and other immune-specific genes that act against *Plasmodium* and prime the mosquito for infection [22]. In this study [114], mosquitoes possessing their midgut microbiota were also shown to have upregulated key antibacterial and anti-*Plasmodium* factors, whereas mosquitoes treated with antibiotics, that eliminate the majority of the midgut microbiota, did not show this upregulation profile and were more susceptible to *Plasmodium* infection. Co-feeding mosquitoes with bacteria and *P. falciparum* gametocytes also resulted in the elicitation of an immune response and resistance to infection. Work by Meister and colleagues has suggested that the PRR molecule known as long peptidoglycan recognition protein C (PGRP-LC), which activates the Imd pathway in the mosquito in response to bacteria, modulates *Plasmodium* infection by controlling the microbial flora in the mosquito midgut [113]. Numerous surveys of mosquito midgut microbiota in laboratory and wild mosquitoes have been performed, and common bacterial genera (*Asaia, Enterobacter*, *Pseudomonas*, *Pantoea*, and others) have been identified, with some of these bacteria being closely associated with *Anopheles* mosquitoes. For example, the acetic acid bacteria *Asaia* has emerged as an important symbiont of *Anopheles* [115]. However, it is not clear if *Asaia* can directly reduce *Plasmodium* infection.

Several studies have shown that the mosquito midgut microbiota negatively affect the ability of *Plasmodium* parasites to develop to the oocyst stage in the mosquito gut tissue [1,116-119]. A number of bacterial species have also been shown to produce potential antimalarial compounds [120], but the effects on mosquito-stage *Plasmodium* development have not yet been examined. Bacteria may play an indirect role in parasite interference through the induction of an anti-*Plasmodium* immune response in the midgut, as discussed earlier.

Recent work by Kumar and colleagues (2010) has revealed a peroxidase/dual oxidase system that forms a dityrosine network in the midgut and decreases the permeability of the midgut to immune activators, protecting the microbiota and also providing a safe environment for *Plasmodium* to develop in the midgut [121]. Dual oxidase (Duox) is a transmembrane protein that produces the hydrogen peroxide substrate for peroxidase. RNAi-mediated silencing of the heme peroxidase-immunomodulatory peroxidase (IMPer) gene has been shown to result in decreased bacterial load in the midgut and induced the upregulation of key antibacterial effectors such as cecropin and PGRP-LB. *IMPer* gene silencing also reduced *P. berghei* ookinete and oocyst development via the induction of NOS in antibiotics-treated (also called aseptic) *An. gambiae* female mosquitoes; in addition, through the induction of NOS, RNAi-mediated silencing of the *IMPer* gene also reduced the development of *P. falciparum* in *An. stephensi* and *An. gambiae* females possessing their microbiota as well in females with decreased microbiota load via treatment with antibiotics. RNAi-mediated silencing of the *Duox* gene also reduced *P. falciparum* in *An. gambiae* via NOS induction. Hence, this complex when intact appears to block midgut immune responses to bacteria and

Plasmodium, allowing proliferation and development.

Although the absolute mechanism by which bacteria inhibit *Plasmodium* is as yet unclear, their potential usefulness as a biologically based control strategy is apparent. A recent study has demonstrated engineered mosquito midgut microbiota potential as a control strategy. In this study, the investigators developed a strategy to engineer symbiotic bacteria to deliver antimalarial effector molecules to the midgut lumen, thereby rendering the mosquitoes resistant to *Plasmodium* infection [122].

Antibacterial effectors

As earlier noted, the mosquito employs antibacterial effectors to battle the malaria parasite. We have discussed some of these effectors earlier in this review with regards to their antiplasmodial roles. In this section, we will briefly highlight some of these effectors' roles in the antibacterial response.

The complement-like protein TEP1 is involved in the bacterial phagocytosis response and has been shown to bind to both G- and G+ bacteria [123]. The mosquito pathogen recognition receptor (PRR) *Ag*Dscam is a determinant of resistance and bacterial phagocytosis and also modulates the mosquito's response to *Plasmodium* infection [72,73]. Fibrinogen-related protein (FREP) FBN9 interacts with G- and G+ bacteria and appears to form dimers in order to specifically bind to bacterial surfaces with different affinities [65]. This FREP may use a multimerization mechanism similar to that of LRIM1/APL1C (earlier discussed in detail) and may dimerize with other FREPs, thereby providing diverse PAMP interaction specificities, as a means of increasing the mosquito's PRR repertoire. Whether FBN9 also forms dimers when binding to human

and rodent malaria parasites remains unknown; however, direct interaction is thought to occur, as implied by FBN9's interaction with bacteria [65]. The PRR GNBPB4 is known to interact with a wide range of pathogens. Particularly, GNBPB4 has been shown to directly interact with *E. coli* and co-localize with *P. berghei* ookinetes [124].

Two other immune-responsive factors involved in the mosquito's antibacterial and antimalarial responses are the *Rel2*-S and *Rel2*-F isoforms of the *Rel2* gene. These isoforms not only modulate the defense against G- and G+ bacteria but also regulate several of the AMPs and antiparasitic genes, as mentioned earlier in this review. The immunoglobulin superfamily (IgSF) members known as the infection-responsive with immunoglobulin domain (*IRID*) genes are factors that also participate in the mosquito's antibacterial and antiplasmodial responses, with the *IRID6* gene functioning to limit *P. falciparum* as well as bacterial infection [125]. Lysozymes, another class of antimicrobial immune effectors, are also important in the antiplasmodial defense. Lysozyme c-1 (LYSC-1) has recently been shown to act as a protective agonist of the development of *P. berghei* and *P. falciparum* oocysts. This antimicrobial effector binds directly to *Plasmodium* oocysts following midgut invasion in *An. gambiae* [126,127]. In addition, silencing of the *LYSC-1* gene in *An. gambiae* as well as in the Asian malaria vector *An. dirus* significantly reduced *P. berghei* infection [127,128].

Concluding Remarks

The *Anopheles* mosquito makes use of many weapons to battle *Plasmodium*. The molecular and cellular events involved in the infection of a mosquito with different *Plasmodium spp.* may be quite similar yet also divergent, indicating the great complexity

and intricacy of parasite-mosquito interactions. Given the increasing prevalence and spread of malaria, especially in Africa, there is an impetus for further dissection of the innate immune system of *Anopheles*, with an emphasis on how it modulates and regulates *Plasmodium* infection. The insight and knowledge gained from such studies can provide the necessary tools for creating antimalarial strategies based on amplifying the mosquito's anti-*Plasmodium* defenses.

Research Objectives

The objectives of this research were to better understand the *Anopheles gambiae* anti-*Plasmodium* response and to understand mosquito-microbiota interactions. Specifically, we aimed at a better understanding of the role of the negative regulator Caudal in the Imd pathway's response to the human malaria parasite *P. falciparum* (Chapter 2). In addition, an assessment of the cross-colonization capacity of the midgut microbiota across the phylogenetically distinct *Anopheles gambiae* and *Aedes aegypti* mosquito species was conducted (Chapter 3).

In this study we aimed to:

AIM 1: Understand the role of Caudal in the *Anopheles* Imd pathway's anti-*Plasmodium* defense response.

Specific Aim 1.1: What is *Cad's* role in the Anopheline anti-*Plasmodium* response?

Specific Aim 1.2: What is *Cad's* role in the regulation of the midgut microbiota and the Anopheline antibacterial defense response?

Specific Aim 1.3: Does manipulation of *Cad* affect Anopheline fitness?

AIM 2: Molecularly assess the cross-colonization capacity of the midgut microbiota

across the phylogenetically distinct *Anopheles gambiae* and *Aedes aegypti* mosquito species.

Specific Aim 2.1: Can Anopheline-derived and/or *Aedes*-derived bacteria isolates colonize the midgut of laboratory *An. gambiae* and/or *Ae. aegypti*?

Specific Aim 2.2: Does the Imd pathway affect cross-colonization capacity of the microbiota in *An. gambiae* and/or *Ae. aegypti*?

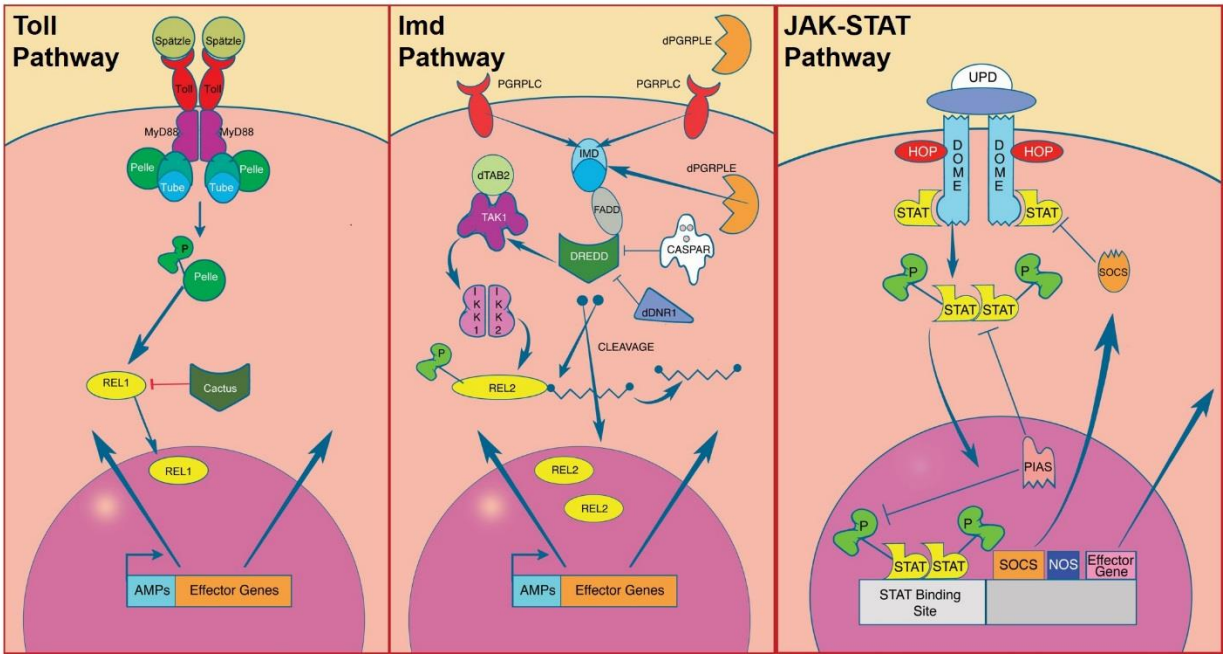

Fig. 1.1. The Toll, Imd and JAK-STAT Immune Signaling Pathways. Upon the recognition of bacteria or *Plasmodium*, the Toll pathway is stimulated by the binding of the ligand Spätzle with the Toll transmembrane receptor. This triggers a series of molecular events that culminate in the activation and translocation of Rel1 into the nucleus, upregulating the transcription of immune genes that are responsible for microbial killing. Numerous studies conducted in *Anopheles* have highlighted the Imd pathway as the most efficient immune pathway in the defense against the human malaria parasite, *Plasmodium falciparum*. The Imd pathway is stimulated when the

transmembrane PGRP-LC receptor recognizes bacteria or *Plasmodium*. This leads to a signaling cascade that will result in the cleavage of Rel2-F and the translocation of active Rel2-S into the nucleus, upregulating the transcription of immune genes. The JAK-STAT Immune Signaling Pathway has been implicated in antibacterial, antiviral, and antiplasmodial defense in mosquitoes. The JAK-STAT pathway is initiated by the binding of the cytokine ligand Unpaired (UPD) to the transmembrane receptor DOME. This then leads to the eventual nuclear translocation of STAT and transcriptional activation of immune effector genes.

CHAPTER 2

CAUDAL IS A NEGATIVE REGULATOR OF THE *ANOPHELES* IMD PATHWAY THAT CONTROLS RESISTANCE TO *PLASMODIUM FALCIPARUM* INFECTION

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Caudal is a negative regulator of the Anopheles IMD pathway that controls resistance to Plasmodium falciparum infection.
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Abstract

Malaria parasite transmission depends upon the successful development of *Plasmodium* in its *Anopheles* mosquito vector. The mosquito's innate immune system constitutes a major bottleneck for parasite population growth. We show here that in *Anopheles gambiae,* the midgut-specific transcription factor *Caudal* acts as a negative regulator in the Imd pathway-mediated immune response against the human malaria parasite *P. falciparum*. *Caudal* also modulates the mosquito midgut bacterial flora. RNAi-mediated silencing of *Caudal* enhanced the mosquito's resistance to bacterial infections and increased the transcriptional abundance of key immune effector genes. Interestingly, *Caudal*'s silencing resulted in an increased lifespan of the mosquito, while it impaired reproductive fitness with respect to egg laying and hatching.

Key words: *Anopheles,* mosquito, *Plasmodium*, innate immunity, *Caudal*, Imd pathway.

Introduction[1]

Malaria, caused by the *Plasmodium* parasite, affects approximately 3 billion people worldwide each year. The major vector for *P. falciparum* in sub-Saharan Africa is the female *Anopheles gambiae* mosquito. Given the lack of an effective vaccine against *Plasmodium* and the increased resistance of this parasite to the current arsenal of drugs and of *Anopheles* mosquitoes to insecticides, the development of novel control strategies is crucial to reducing malaria transmission [1].

Malaria transmission between humans depends on the successful completion of the parasite's lifecycle in the mosquito. This journey begins with the ingestion of *Plasmodium* gametocytes by the female mosquito through a blood meal. The *Plasmodium* male and female gametocytes differentiate in the mosquito's midgut lumen, where fertilization produces zygotes. The zygotes are transformed into motile ookinetes that invade and migrate across the midgut epithelium at roughly 18-36 h post-ingestion. Once

[1] ***Abbreviations:*** *Cad*, *Caudal*; *Pf*, *Plasmodium falciparum*; Imd, immune deficiency; RNAi, RNA interference; dsRNA, double-stranded RNA; CFU, colony-forming unit; ROS, reactive oxygen species; Cp, carboxypeptidase; ANK, ankyrin; TEP1, thioester-containing protein 1; FBN9, fibrinogen immunolectin 9; APL1, *Anopheles Plasmodium*-responsive Leucine-rich repeat protein 1; APL2, *Anopheles Plasmodium*-responsive Leucine-rich repeat protein 2; LRIM1, Leucine-Rich Immune Molecule 1; AMPs, antimicrobial peptides; *Cec-1*, *Cecropin-1*; *Cec-3*, *Cecropin-3*; *Def-1*, *Defensin-1*; *Gam*, *Gambicin*.

the ookinetes have reached the basal side of the midgut epithelium, they transform into oocysts and subsequently undergo several rounds of mitosis. The oocysts then rupture, releasing thousands of sporozoites into the mosquito hemocoel about 14 days after the infectious blood meal. The sporozoites migrate through the mosquito hemolymph and invade the salivary glands. During the female *Anopheles'* next blood meal, these *Plasmodium* sporozoites are injected into the human host, thereby completing the sexual cycle within the mosquito vector (reviewed in [13,14]).

The innate immune system of *Anopheles,* the vector's main line of defense against parasites, fungi, bacteria, and viruses, is engaged at multiple stages of *Plasmodium* infection [13,22-24]. Much insight concerning this innate immune defense has been gathered from studies in *Drosophila* (reviewed in [24,25]). The mosquito's anti-*Plasmodium* and antibacterial defenses are largely controlled by the Toll and Imd (immune deficiency) NF-kappaB immune signaling pathways. Infection-responsive activation of the Toll and Imd pathways ultimately leads to the nuclear translocation of the NF-kappaB transcription factors *Rel1* and *Rel2*, respectively. These transcription factors are negatively regulated in the cytoplasm by *Cactus* and *Caspar,* respectively. Elicitation of the Toll and Imd pathways causes the degradation of *Cactus* and *Caspar*, allowing the *Rel* factors to enter the nucleus and transcriptionally activate immune effector genes such as antimicrobial peptides (AMPs) and other factors. Through alternative splicing, the *Rel*2 gene produces a full-length form (*Rel*2-F) that includes the carboxyl-terminal ankyrin (ANK) and death domains, and a shorter form (*Rel*2-S) lacking such domains. The *Rel2*-S form is constitutively translocated to the nucleus, where it regulates the transcription of immune genes [27].

While the Toll pathway has been shown to defend against *P. berghei* and *P. falciparum*, the Imd pathway has emerged as the most effective pathway in the defense against the human malaria parasite *P. falciparum* [30-33]. Specifically, investigations from our lab demonstrated that depletion of *Caspar*, a negative regulator of *Rel2*, results in an Imd pathway-mediated immune defense that confers almost complete refractoriness to *P. falciparum* in three major *Anopheles* malaria vector species: *An. gambiae*, *An. stephensi*, and *An. albimanus*. Depletion of the Toll pathway negative regulator *Cactus* resulted in a significantly greater resistance to infection with the rodent malaria parasite *P. berghei* [31]. Our previous work has also shown that these two *Plasmodium spp.* elicit diverse innate immune responses at the gene transcript level [34]. Previous studies conducted by us and by others have identified a diverse repertoire of anti-*Plasmodium* immune effectors regulated by *Rel2*, including APL1, TEP1, LRRD7 (APL2), FBN9, and LRIM1 [27,33-37,129,130]. We have also substantiated and characterized the potency of the Imd pathway in the anti-*Plasmodium* defense through the use of genetically modified immune-enhanced *Anopheles* mosquitoes that express blood meal-inducible *Rel*2 in both the midgut and fat body tissues [40]. The transient activation of this transgene resulted in almost complete resistance to the human malaria parasite at a negligible fitness cost, prompting further investigation of this system as an innovative malaria control strategy [40]. We have also shown that the natural bacterial flora of the mosquito midgut elicits a basal level of immune activity mediated by AMPs and other effector genes that act against *Plasmodium* and thus primes the mosquito for infection [114].

The potency of the Imd pathway in the anti-*P. falciparum* response warrants further dissection in light of recent studies [31,32,40]. There are, for example, only a few studies

detailing the regulation of *Rel2* once it has been translocated to the nucleus. Here, we show that the transcription factor *Caudal* (*Cad*) is an antagonist of *Rel2,* and thus a negative regulator of the Imd pathway in the *Anopheles* mosquito. While our studies support previous work performed in adult *Drosophila*, we show for the first time a role for *Cad* in the immunity of the adult *Anopheles* mosquito to *Plasmodium* infection and its role in influencing Anopheline fitness.

Findings in *Drosophila* identify *Cad* as a homeobox transcription factor that regulates posterior and intestinal development of the *Drosophila* embryo, and it has also been shown to be required for embryogenesis in nematodes, other arthropods, and vertebrates [131-138]. *Cad* in adult *Drosophila* is predominantly expressed in the posterior midgut, where it is involved in the maintenance of midgut-specific gene transcription [139,140]. Ryu and colleagues [42] showed that silencing *Cad* led to the overexpression of certain AMPs and a modulation of the midgut microbiota. Their study emphasized the antagonistic relationship between *Cad* and *Relish,* a relationship that ensures a balance between immune defense and microbiota homeostasis in the gut [42,141-144].

In our study, RNAi-mediated silencing of *Cad* specifically compromised *P. falciparum* development in the gut tissue, suppressed the midgut microflora, and enhanced resistance to systemic bacterial infections, most likely by causing an increased transcriptional abundance of AMPs and other effector genes. Interestingly, *Cad* silencing resulted in increased longevity, but it impaired the mosquitoes' fecundity and fertility.

Materials and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Care and Use Committee of the Johns Hopkins University (Permit Number: M006H300). Commercial anonymous human blood was used for parasite cultures and mosquito feeding, and informed consent was therefore not required. The Johns Hopkins School of Public Health Ethics Committee has approved this protocol.

Mosquito rearing

An. gambiae Keele strain mosquitoes were maintained under laboratory conditions at 27°C and 80% humidity with a 12 h day-night cycle. Larvae were reared on cat food pellets and ground fish food supplement. Adult mosquitoes were maintained on 10% sucrose and fed on mouse blood (mice were anesthetized with ketamine) for egg production [145].

RNA isolation, quantitative real-time PCR (qRT-PCR), and RNA interference (RNAi)-mediated gene silencing

RNA was extracted and quantified (in triplicate samples) in different tissues (whole mosquito, gut, and fat body) using the RNeasy kit (Qiagen), and cDNA was prepared using the oligo(dT_{20}) primer and the Invitrogen Superscript III reverse transcriptase according to standard methodology. The quantitative real-time PCR (qRT-PCR) and RNAi gene-silencing assays were carried out according to [34], and the ribosomal protein *S7* gene was used for normalization of the cDNA templates. The fold change in the gene

expression and the gene silencing efficiency (from the RNAi assays) were calculated according to the standard $E^{\Delta\Delta Ct}$ method [146] when both primer efficiencies of the GOI (gene of interest) and the *S7* gene were equal. The primer efficiencies were determined as described in [146].

Several *An. gambiae* immune genes were screened for either anti-*Plasmodium* or antibacterial defense activity using RNA interference (RNAi) in wildtype mosquitoes. For these assays, the mRNA for the specific gene was selectively depleted from the adult female mosquitoes using established RNAi methodology [147]. The *dsRNA* injection assay of different genes was repeated three independent times with at least 50-80 mosquitoes in each experiment; the *GFP dsRNA*-injected mosquitoes served as controls. The RNAi gene silencing efficiencies were determined at 2-3 d post *dsRNA* injection for all genes tested and compared to the *GFP dsRNA*-injected control mosquitoes. The primers used for *dsRNA* synthesis and silencing verification, together with the gene silencing efficiencies, are presented in Supplementary Table 1 (Table S1). Additionally, a time course of the gene silencing efficiency of *Caudal* is represented in Supplementary Figure 1.

P. falciparum and P. berghei infection assays

To determine anti-*Plasmodium* activity, mosquitoes were fed on NF54 *P. falciparum* gametocyte cultures (0.01-0.05%, 0.05-0.1%, or 0.1-0.5% gametocytemia) (provided by the Johns Hopkins Malaria Institute Core Facility) through artificial membranes at 37°C or on a *P. berghei* ANKA-infected Swiss Webster mouse (at 19°C) [34]. The adult mosquitoes were starved for 8 to 12 h prior to feeding to ensure engorgement. To

determine oocyst numbers, unfed mosquitoes were first removed, and the remaining mosquitoes were incubated for a further 7 days at 27°C or 13 days at 19°C for *P. falciparum* or *P. berghei*, respectively. Midguts were dissected out in PBS, stained with 0.2% mercurochrome, and examined using a light-contrast microscope (Olympus). Three biological replicates were used in each experiment.

A previously described method [116] was used to determine the sporozoite loads in the salivary glands of the infected mosquitoes. In brief, salivary glands were dissected; individual glands were then placed in Eppendorf tubes with 120 ul of PBS and homogenized (on ice). The homogenate was centrifuged at 8,000 rpm for 10 min, and approximately 90 ul of the supernatant was removed. The sporozoites were resuspended in the final 30 ul of PBS, and 10 ul of this suspension was placed in a Nuebauer counting chamber and counted after 10 min with a Leica phase-contrast microscope at 400x magnification.

The dot plots of the oocyst and sporozoite numbers in each gut epithelium and salivary gland, respectively, for each treatment were generated using the GraphPad Prism5 software, along with the median value indicated. Statistical significance indicated on the dot plots was derived from the Mann-Whitney Test. Additionally, when necessary, the Kruskal-Wallis test was performed with a Dunn's multiple comparison post-test. See Table S2 for statistical analyses and prevalence rates of oocyst or sporozoite loads in dsRNA-treated mosquito midguts or salivary glands, respectively. Statistical significance was denoted as follows: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

Characterization of proliferated midgut microbial flora and Bacterial challenge survival assays

Isolation and colony-forming unit (CFU) enumeration of endogenous gut bacteria from 2-3 d old *Cad* and *GFP* dsRNA-treated mosquitoes were performed as previously described [114]. Briefly, mosquitoes were surfaced-sterilized by dipping them in 100-70% EtOH for 2 min and then rinsing them with 1X PBS twice for 1 min each. Midguts were dissected from each individual mosquito over a sterile glass slide containing a drop of 1X PBS, then transferred to a microcentrifuge tube containing 150 ul of sterile 1X PBS, and macerated for 30 sec. Three 10-fold serial dilutions were then plated on LB agar and kept at room temperature for 48 h. Initial isolation was based on morphology, color, and size of the colony and was followed by molecular identification via *16S* rDNA sequencing. Primers used to amplify the *16S* rDNA were: Forward, AGAGTTTGATCCTGGCTCAG; Reverse (degenerate), TACGGYTACGCTTGTTACGACT [116].

Survival assays were conducted following bacterial challenges of *Cad*-, *Cactus*-, *Caspar*-, and *GFP* dsRNA-injected mosquitoes according to established protocols [34,148] with minor modifications. In brief, at 3-4 d post-injection, mosquitoes were injected in the thorax via a nano-injector (Nanoject, Drummond) with 69 nl of bacterial suspension (OD_{600} 3.5 of *E. coli* –approximately $5x10^{15}$ cells/mL- or OD_{600} 0.8 *S. aureus*- approximately $7x10^{15}$ cells/mL). For negative controls, 69 nl of 1X PBS was injected into control and experimental group mosquitoes. Dead mosquitoes were counted daily over a 7 d period after challenge with bacteria or 1X PBS. Approximately 20-30 mosquitoes

were used for each group of injected mosquitoes, and three replicates were performed for all experiments. Survival patterns were determined using Kaplan-Meier survival analysis, and a log-rank test was used for significance evaluation with the GraphPad Prism5 software as described in [72]. To clarify, the overall significant trend refers to the log rank test result of the Kaplan-Meier analysis. The log rank test compares survivorship among experimental groups to the control group. The analysis used did not measure if the experimental groups were significant from each other. See Table S3 for Kaplan-Meier survival analyses of dsRNA-treated mosquitoes after systemic bacterial infections.

Longevity, fecundity, and fertility assays

For the longevity assays of mosquitoes maintained on 10% sucrose solution only, approximately 30 to 80 3-4 d old adult female *Cad* and *GFP* dsRNA-treated mosquitoes were kept in a wax-lined cardboard cup at 27°C with 70% humidity. In a second cohort, the mosquitoes' survival rate was also monitored by providing a single naïve human blood meal to 3-4 d old dsRNA-treated mosquitoes, which were then maintained on a 10% sucrose solution. Alternatively, a cohort of 3-4 d old dsRNA-treated mosquitoes were provided with a *Pf*-infected blood meal (0.01-0.05% gametocytemia) and then maintained on a 10% sucrose solution. Three independent experiments were performed for each cohort, and all cohorts were monitored daily for survival. Monitoring continued until all mosquitoes had perished. The survival percentage represents the mean survival percentage for all three biological replicates as described in [31,40]. Kaplan-Meier survival analysis and log-rank tests were performed as previously described and are available in Table S4.

For the fecundity assays, approximately 40-90 3-4 d old *Cad* and *GFP* dsRNA-injected and uninjected mosquitoes were allowed to feed on human blood through an artificial membrane feeder for 30 min. The fed mosquitoes were transferred to individual vials (one mosquito per vial) outfitted with cotton and with moistened filter paper at the bottom of the vial. Individual chambers were incubated under normal rearing conditions. Eggs oviposited on filter paper were counted after 2 d using light microscopy. Female mosquitoes that did not produce eggs on day 2 were maintained and re-examined on day 3. After each count, eggs were submerged in a standard larval pan for rearing according to standard methods. First- to second-instar larvae were counted to determine the larval hatch rate. The fecundity and larval hatch-rate assays were performed on three biological replicates, and the number of eggs laid by each female and the respective hatch rates were used to calculate mean values. Statistical significance was determined using the Mann-Whitney test. Statistical analyses are available in Table S5.

Results

Caudal is a midgut-specific transcriptional regulator of immune effector genes

In *Drosophila*, *Cad* is a developmental transcription factor as well as a midgut-specific antagonist of the Imd pathway transcription factor *Relish* [42,140]. Because the innate immune signaling pathways are highly conserved between *Drosophila* and *Anopheles* [68], we hypothesized that *Cad* (Gene ID: AGAP009646) might play a similar role in *An. gambiae*. To test this hypothesis, we assayed *Cad* transcript abundance in the adult female mosquito midgut, fat body, and remaining tissue (carcass). Consistent with the expression pattern observed in adult *Drosophila* [42,139,140], we respectively saw an

almost 18-fold higher abundance of *An. gambiae Cad* transcripts in the fat body and a ~100-fold higher abundance in the midgut than in the carcass (Fig. 2.1A).

We next hypothesized that mosquito *Cad* also negatively regulates gene expression by the Imd pathway transcription factor *Rel2*. To test this hypothesis, we analyzed mRNA levels of the *Rel2*-regulated antimicrobial peptide (AMP) genes *Cecropin-1* (*Cec-1*), *Cecropin-3* (*Cec-3*), *Defensin-1* (*Def-1*), and *Gambicin* (*Gam*) and the anti-*Plasmodium* effector genes *FBN9* and *LRRD7* at 12 h and 24 h in the midgut following RNAi-mediated silencing of *Cad* as compared to mRNA levels in *GFP* dsRNA-treated mosquito midguts (Fig. 2.1B). This panel of effector genes represents the three major classes of AMPs and anti-*P. falciparum* effectors, and they are all regulated by microbial challenge [27-29,34,71,147,149-152] (Fig. 2.1B). An approximately 2-fold increase in the transcript abundance of *Cec-1*, *Cec-3*, and *Def-1* was observed at 12 h after *Cad* silencing, while the abundance of *Gam* transcripts decreased by almost 2-fold. At 24 h after *Cad* silencing, we observed a 2- to 3-fold change in the abundance of *Def-1*, *Cec-1*, *Cec-3*, and *Gam* transcripts. The anti-*Plasmodium* effectors *FBN9* and *LRRD7* displayed a ~2-fold increase in transcript abundance at 12 h after *Cad* silencing, but they were suppressed to almost the same degree at 24 h after silencing. The down regulation observed for these two effectors may be explained by complex temporal transcriptional regulation. Additionally, as a transcription factor, *Cad* may function in multiple pathways. Similar dynamic profiles of immune gene regulation has been observed in which certain effectors are up regulated while others are down regulated at a specific time point in previous studies where components of the Imd-*Rel2* pathway were manipulated in *Anopheles* [31,40].

Caudal controls P. falciparum but not P. berghei infection

Since *Cad* is a midgut-specific transcriptional modulator of immune effector genes (Fig. 2.1B), we subsequently investigated the effect of its silencing on the mosquito's susceptibility to *P. berghei* and *P. falciparum* infection of the midgut. The median number of *P. falciparum* oocysts in *Cad*-silenced mosquitoes was significantly lower (~3-fold) than in *GFP* dsRNA-injected controls at 7 days after feeding on a gametocyte culture (Fig. 2.2A). However, the median number of oocysts in *Cad*-silenced mosquitoes infected with the rodent parasite *P. berghei* (Fig. 2.2B) was not significantly different from that of control *GFP* dsRNA-treated mosquitoes.

Our data indicate that the negative regulation of innate immune responses by *Cad* affects *Plasmodium* development in a pattern similar to the Imd pathway (Fig. 2.2A, B) [31-33,40]. To further investigate *Cad's* relationship with the Imd and Toll pathway-mediated defenses against *Plasmodium* infection of the midgut tissue, we co-silenced *Cad* with factors of these two pathways and assessed the effect of this silencing on parasite infection (Fig. 2.2C, D). Co-silencing of the Toll pathway transcription factor *Rel1* with *Cad* did not influence the effect of *Cad* silencing on *P. falciparum* infection (Fig. 2.2C). In contrast, co-silencing of the Imd pathway transcription factor *Rel2* reverted the effect of *Cad* silencing on mosquito resistance to *P. falciparum* infection, indicating an antagonistic relationship between these factors and implicating *Cad* as a negative regulator of the Imd pathway (Fig. 2.2C). The influence of *Cad* silencing on the resistance to *P. falciparum* followed a similar pattern to that produced by depleting *Caspar*, a negative regulator of the Imd pathway upstream of *Rel2*-mediated transcription [31,32] (Fig. 2.2D). Co-silencing of *Cad* with *Imd* also minimized the effect of *Cad*

silencing on *P. falciparum* infection, further corroborating a role for *Cad* as a negative regulator of the Imd pathway (Fig. 2.2D). We do note that interpretation of RNAi co-silencing assays needs to be done cautiously since co-silencing phenotypes can depend on a variety of parameters such as gene silencing efficiency and mRNA and protein turnover. Further information on the statistical analyses and prevalence rates are provided in Table S2.

To gain a better understanding of *Cad*'s influence on the mosquito's overall susceptibility to *P. falciparum*, we investigated sporozoite loads in the salivary glands of *Cad*-silenced mosquitoes (Fig. 2.2E). We saw a ~3-fold decrease in *P. falciparum* sporozoite load in the salivary glands of *Cad*-silenced mosquitoes relative to control mosquitoes (Fig. 2.2E). It is important to note that the data presented in Fig. 2.2A and Fig. 2.2E is not from the same infection experiment (supplemental information provided in Table S2). Although silencing of *Cad* did not render mosquitoes completely refractory to *P. falciparum* at high infection intensities (Fig. 2.2A-2E), it is important to note that laboratory infection levels are much higher than those in nature. We have previously shown that manipulation of the Imd components *Caspar* and *Rel2* can render mosquitoes refractory when infection levels are similar to those observed in the field [32,40].

Caudal -mediated immune activation potentiates transgenic resistance to Plasmodium

We have shown that genetic manipulation of the Imd pathway can confer resistance to *P. falciparum* infection through the transient overexpression of *Rel2* [40]. Our findings suggest that RNAi-mediated silencing of *Cad* could be used to further potentiate the anti-*Plasmodium* response produced by the overexpression of the transgene *Rel2*. To investigate this possibility and to better understand the function of *Cad* in modulating

Rel2-controlled anti-*Plasmodium* activity, we assessed the impact of *Cad* silencing in transgenic *An. stephensi* mosquitoes that overexpressed *Rel2* after a blood meal in the midgut (the Cp-carboxypeptidase- line) [40]. We performed these assays at infection intensities similar to those observed in the field [153]. Silencing the *An. stephensi Cad* gene resulted in a significantly lower median number of oocysts in wildtype as well as transgenic mosquitoes when compared to *GFP* dsRNA-treated control mosquitoes (Fig. 2.2F). Interestingly, *Cad* silencing rendered both WT and transgenic mosquitoes refractory to *P. falciparum* infection as a measure of median oocyst load. These results demonstrate that *Cad* plays a conserved role in the anti-*P. falciparum* defense across two malaria vector species and that it can further boost the anti-*P. falciparum* activity conferred by transgenic *Rel2* overexpression. Hence, *Cad* can potentially be used to increase resistance to *P. falciparum*, and it may therefore represent a promising immune factor for use in developing novel malaria control strategies based on genetically engineered *Plasmodium*-resistant mosquitoes.

Caudal regulates the proliferation and species composition of the mosquito's midgut microbiota

While the mosquito midgut microbiota is beneficial and necessary for the insect, it can become detrimental if it over proliferates, and it must therefore be kept in check by the innate immune system [117]. The Imd pathway has been shown to play an active role in this process as a key regulator of immune responses in the *Drosophila* and mosquito gut [113,154], and we have already shown that *Cad* influences AMP transcript abundance by negatively regulating the Imd pathway, a finding that suggests an active role for *Cad* in the suppression of the midgut microbiota (Fig. 2.1B). In the present study, we also

investigated the influence of *Cad* silencing on the prevalence and species composition of the midgut microbiota. We first focused on LB-culturable bacteria, and we observed that silencing *Cad* produced a significant decrease (by ~6-fold) in the average midgut bacterial load when compared to *GFP* dsRNA-treated control mosquitoes, and it resulted in the modification of the bacterial species composition (Fig. 2.3A; Supplementary Tables S6 and S7). Specifically, a decrease in overall species diversity (based on *16S* rDNA sequences) from seven to three Gram-negative bacterial species was observed upon *Cad* silencing.

To provide information concerning the influence of *Cad* on the majority of the total microbiome, we also investigated the result of *Cad* silencing on bacterial load by conducting qRT-PCR analysis of *16S* rRNA gene expression in the midgut over a time course of 12, 24, and 48 h after *Cad* dsRNA treatment compared to control mosquitoes treated with *GFP* dsRNA. Our data showed (Fig. 2.3B) that *16S* rRNA abundance was decreased at 12 (~8-fold), 24 (~2-fold), and 48 h (~12-fold) after *Cad* dsRNA injection. The diminished proliferation of the midgut microbiota in response to *Cad* silencing is likely attributed to the increased transcriptional abundance of *Rel2*-controlled AMP and other effector genes (Fig. 2.1B). Such dynamics could reflect the differential proliferation among, and competition between, different bacterial species in response to an enhanced immune response mediated by the silencing of *Cad*. Since silencing of *Cad* depletes the microflora, which has been shown to act negatively on *Plasmodium*, this decreased number of gut microbes may therefore partly offset the enhanced anti-*Plasmodium* immune defense upon *Caudal* silencing.

Caudal's role in the defense against systemic bacterial infections

We continued our investigations of *Cad*'s role in antibacterial defense by investigating its role in systemic bacterial infections. We assessed the survival of *Cad*-, *Cactus-,* or *Caspar*-silenced and *GFP* dsRNA-treated control mosquitoes after systemic infection with either *E. coli* (Gram-negative) or *S. aureus* (Gram-positive). The two negative regulators *Cactus* and *Caspar* were also included in these experiments, since the Toll pathway has been shown to be primarily involved in the defense against Gram-positive bacterial infections, while the Imd pathway primarily acts against Gram-negative as well as some Gram-positive bacteria [22,27,31]. We assayed mosquito mortality for 7 days after challenge via intra-thoracic injections with either bacteria or with 1X PBS (as a control--data not shown) (Fig. 2.3C, D). Silencing of the three immune factors after *S. aureus* challenge resulted in quite different mosquito survival patterns (Fig. 2.3C), and an overall significant trend was observed. As expected, *Cactus* silencing increased mosquito survival after Gram-positive bacterial infection. Interestingly, *Cad* silencing also potentiated the mosquito's ability to defend against Gram-positive bacterial challenge to a greater degree than *Caspar* silencing or *GFP* dsRNA treatment of mosquitoes. This could be explained by the observed increase in elicitation of *Def-1* and *Gam* (Fig. 2.1B), which have previously been shown to participate in the defense against Gram-positive bacteria (*Def-1*) or against both bacterial classes and also *Plasmodium* (*Gam*) [34,151]. Alternatively, the extended lifespan could reflect some other function of *Cad* that influences mosquito longevity. While silencing of the three immune factors did not result in different survival patterns after *E. coli* challenge, we noted that the mosquitoes in the *Cad*-silenced group were able to survive longer after challenge than the other experimental groups or the controls (Fig. 2.3D). This difference could be due to the

increased transcript abundance of *Cec-1* and *Cec-3*, which have been implicated in the defense against Gram-negative bacteria [29,149,150,152,155]. *Cad* confers resistance to bacterial infection possibly via increasing the transcript abundance of AMPs, a finding corroborated by other recent and relevant studies [40,156]. Since *Cad* is midgut-specific, our data also suggest that this tissue can play a role in systemic immune responses against pathogens in the insect's hemocoel.

Caudal's effects on mosquito fitness as a measure of longevity, fecundity, and fertility

Studies of the trade-offs between lifespan and immune defense have shown that mounting an immune response may accelerate aging in insects; chronic and sustained, but not acute and transient, *Relish*-dependent immune signaling reduces the lifespan of *D. melanogaster* and *Anopheles* [31,40,157,158]. To provide further insight on the biology of *Cad* in *An. gambiae*, we assessed the impact of its transient silencing on several facets of mosquito fitness. Specifically, we monitored longevity, fecundity (number of eggs per female), and fertility (number of eggs that hatch into larvae) in *Cad* and *GFP* dsRNA-treated mosquitoes. We assessed longevity in dsRNA-treated mosquitoes that had been maintained under one of the three following conditions: 1) on 10% sucrose only, 2) fed a naïve blood meal and then maintained on 10% sucrose, or 3) fed on a *Pf*-infected blood meal and then maintained on 10% sucrose (Fig. 2.4A). Surprisingly, the silencing of *Cad* increased the longevity of mosquitoes under all three conditions, with an overall significant trend. Particularly, *Cad* dsRNA-treated mosquitoes that had obtained a naïve blood meal and were then maintained on 10% sucrose survived longer than the other experimental groups (Fig. 2.4A). Such a conferred fitness advantage of a *Plasmodium*-resistant phenotype upon blood feeding is particularly interesting from a malaria control

perspective. This increased longevity could be due to the fact that *Cad* results in a lower prevalence of specific midgut-associated bacterial species that may proliferate greatly after blood meal ingestion and negatively affect mosquito longevity, or it may be the result of some other unknown function of *Cad* that is not necessarily related to the immune system.

In contrast, silencing of *Cad* impaired both the fecundity and fertility of mosquitoes (Fig. 2.4B, C). Approximately 50% of the *Cad*-silenced mosquitoes failed to produce eggs after a naïve blood meal, and the overall egg production by this cohort was 33% and 45% less than that of the *GFP* dsRNA-treated and untreated mosquito control cohorts, respectively (Fig. 2.4B). Also, eggs produced by *Cad*-silenced mosquitoes displayed a 45% and 41% lower hatch rate than that of eggs produced by the *GFP* dsRNA-treated and untreated mosquitoes, respectively (Fig. 2.4C). *Caudal*'s impact on fitness is in contrast to the results of our previous studies on the Imd pathway, which did not indicate any such impairment of fecundity or fertility. Specifically, *Caspar*-silenced or *Rel2*-overexpressing mosquitoes showed no or only minimal differences in longevity, fecundity, and fertility compared to control mosquitoes [31,40]. The differential effects of *Cad* silencing on fitness, as compared to those of other Imd pathway factors, may highlight the functional versatility of this factor in the mosquito, since *Caudal* was first identified as a gene involved in *Drosophila* embryonic posterior development [131,135].

Discussion

Caudal is a regulator of the Imd pathway-controlled transcription factor Rel2

In this study, we show that *Cad* is a midgut-specific antagonist of the Imd pathway transcription factor *Rel2*. Silencing of *Cad* resulted in a general increase of *Rel2*-

regulated AMP and anti-*Plasmodium* effector gene mRNA abundance (Fig. 2.1B) in a manner similar to when the Imd pathway is activated through other means of molecular manipulation in *Anopheles* [31,40].

Caudal regulates Plasmodium species-specific defense

We demonstrate that *Cad* specifically controls resistance to *P. falciparum* and not *P. berghei* infection in a pattern similar to the Imd pathway (Fig. 2.2A-2D) [31-33,40]. Silencing of *Cad* decreased oocyst loads of the midgut and sporozoite loads in the salivary glands, thereby influencing the mosquito's overall vector competence for *P. falciparum* (Fig. 2.2E). We also show that RNAi-mediated silencing of *Cad* can further potentiate the anti-*Plasmodium* response produced by the overexpression of a recombinant *Rel2* in transgenic *An. stephensi* (Fig. 2.2F). This demonstrates that *Cad* plays a conserved role in the anti-*P. falciparum* defense across two malaria vector species. *Cad* may therefore represent a promising immune factor for use in developing novel malaria control strategies based on genetically engineered *Plasmodium*-resistant mosquitoes.

Caudal regulates microbial homeostasis of the midgut

Our studies demonstrate an active role for *Cad* in the regulation of the midgut microbiota (Fig. 2.3A, 3B). Particularly, *Cad* silencing resulted in a decreased prevalence and altered species composition of the midgut microbiota. The diminished proliferation of the midgut microbiota in response to *Cad* silencing is likely attributed to the increased transcriptional abundance of *Rel2*-controlled AMP and other effector genes (Fig. 2.1B).

Cad also plays a role in the systemic antibacterial defense with regards to positively influencing survival against hemolymph bacterial infections (Fig. 2.3C, D).

Caudal's influence on mosquito fitness suggests functional diversity

We have previously shown that a transient activation of the Imd pathway-controlled *Rel2* did not significantly impact longevity, fecundity and fertility. In contrast, silencing of *Cad* increased the longevity (Fig. 2.4A) and impaired both the fecundity and fertility of mosquitoes (Fig. 2.4B, C). *Caudal*'s impact on fitness is in contrast to the results of our previous studies on the Imd pathway and suggests functional versatility of this factor in the mosquito. *Drosophila Caudal* was first identified as a gene involved in embryonic posterior development [131,135]. With respect to embryogenesis, *Caudal* is expressed during *Drosophila* oogenesis and blastoderm cellularization [135]. Also, maternal expression of *Caudal* in the ovarian follicles has been reported in the dipteran flies *Lonchoptera, Empis,* and *Haematopota* [159]. Previous work has highlighted *Caudal*'s role in regulating the specification and organization of the genital disc in *Drosophila*. The genital disc originates from a portion of the embryonic tail segments and eventually manifests as adult female or male genitalia structures and the analia structures [160-162]. Our collective knowledge of *Caudal*'s developmental functions in *Drosophila* and other dipteran insects points to the likelihood of similar roles for *Caudal* in *An. gambiae* and may also explain why silencing of this gene impairs egg production and larval hatching.

Interestingly, the effect of *Cad* silencing on adult mosquito fitness parameters is also significantly contrasted with those imposed by *Cad* silencing in adult *Drosophila* [42]. As compared to wildtype flies, *Cad*-silenced flies displayed a greater frequency of

midgut cell apoptosis, which in turn negatively affected longevity [42]. The physiology of the hematophagous mosquito gut differs greatly from that of the fly, and this difference could explain the discrepancy of the results in the two insects. However, it would be interesting to investigate whether any apoptosis is occurring in *Cad*-silenced mosquitoes that could impose reproductive costs rather than being a detriment to the mosquitoes' lifespan. Previous studies in *Anopheles* have shown that immune elicitation in response to bacterial and *Plasmodium* infections negatively affects reproductive capability by causing cell death in the ovarian follicular epithelial cells, thereby decreasing oocyte development [163-165]. Pathogen infection-induced reactive oxygen species (ROS) have also been shown to impair reproduction by causing oocyte damage [166,167]. It would therefore also be interesting to explore the relationship between ROS production and *Cad* silencing with respect to reproductive fitness.

The negative impact of *Cad* silencing on reproduction could alternatively be the result of a reallocation of energy during a time of need to combat infection. For example, in *Drosophila*, the fat body has been shown to reallocate resources in order to elicit an immune response to infection and, as a result, suppresses insulin signaling and diminishes nutrient stores and growth [168]. The dynamic shift between immunity and reproduction output observed in our study is consistent with a recent study by Rono and colleagues [64], which revealed that two nutrient proteins crucial for oocyte production, lipophorin and vitellogenin, interfere with the anti-*Plasmodium* effector TEP1. This group also showed that the Imd and Toll pathways inhibit the expression of vitellogenin in order to confer immune defense against *Plasmodium*. *Caudal* could play a key role in this molecular mechanism that determines the trade-off between the anti-*Plasmodium*

response and oocyte development and production.

In sum, our findings show that *Anopheles Cad* functions in the Imd pathway to significantly modulate the mosquito's anti-*P. falciparum* defense. It is also a key factor in the mosquito's antibacterial defense and regulates the species composition and load of the mosquito midgut microbiota. Although we observed a fitness trade-off as a result of *Cad* silencing, the negative impact on mosquito fecundity and egg hatch rate may be overcome via an effective gene drive mechanism. The increased longevity may also allow mosquitoes to engage in additional gonotrophic cycles, and this may compensate for an impaired reproductive fitness compared to their wildtype counterparts [169-171]. In conclusion, our study encourages further investigation of *Cad*'s use as a tool for modulating the ability of mosquito vectors to transmit malaria.

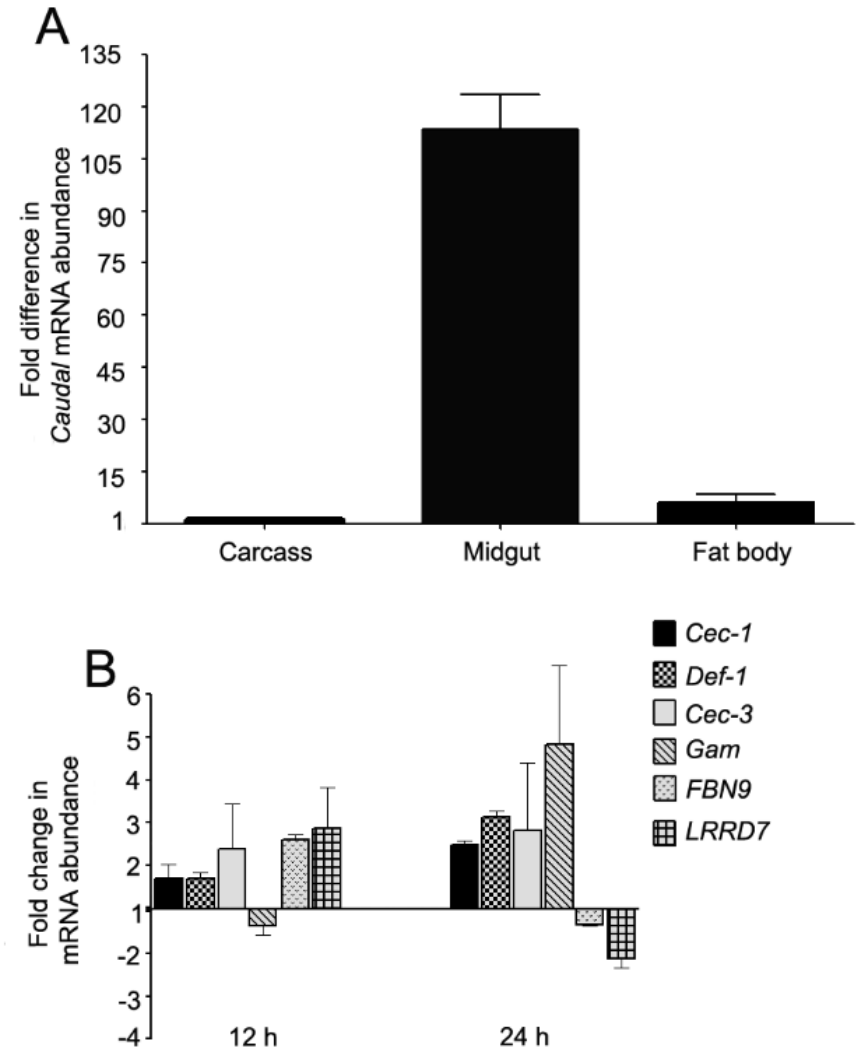


Fig. 2.1. *Caudal* expression in *Anopheles gambiae* and Immune-transcriptional profile upon *Caudal*-silencing. (A) Transcript abundance of *Cad* in the carcass, midgut, and fat body of wildtype female mosquitoes (ribosomal gene *S7* used as an internal control). To clarify, this data represents the expression of *Cad* in different tissues relative to the expression of *Cad* found in the carcass of the mosquito (set at a basal level of 1). (B) Midgut-specific transcript abundance of select AMP and anti-*Plasmodium* effector genes (with ribosomal gene *S7* used as an internal control) at 12 and 24 h post RNAi-mediated silencing of *Cad*. Each column and error bar represents the fold change and the

standard error of the mean compared to *GFP* dsRNA-treated mosquito midguts. For both figures, three biological replicates were performed.

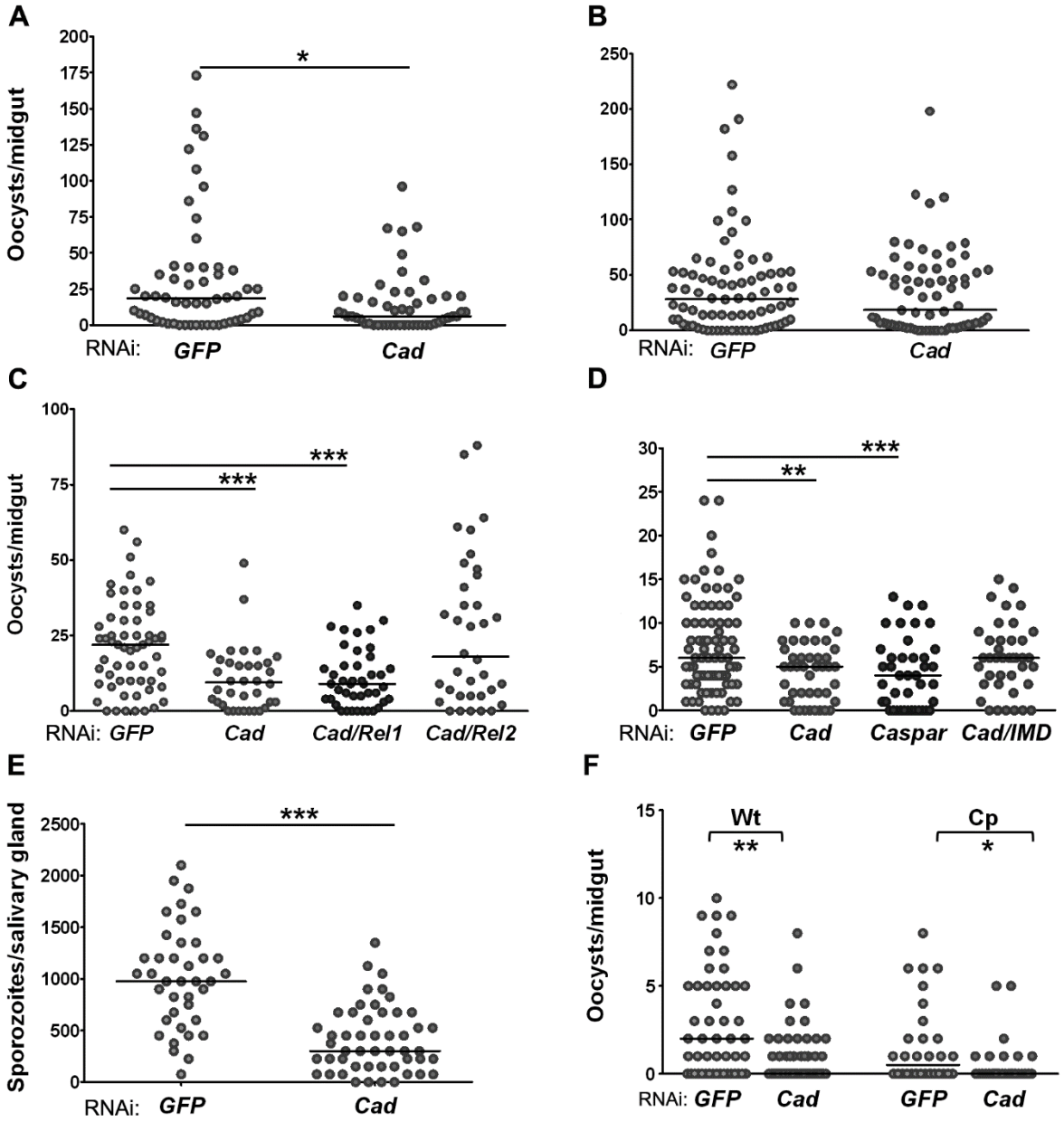


Fig. 2.2. Anti-*Plasmodium* defense in *Caudal*-silenced mosquitoes. *P. falciparum* oocyst (A) and *P. berghei* oocyst (B) loads in the midguts of *An. gambiae* mosquitoes

silenced for *Caudal* (*Cad*) or injected with dsRNA *GFP* (control). (C-D) *P. falciparum* oocyst loads in the midguts of *An. gambiae* mosquitoes silenced for *Cad* or *Caspar* (D) as well as mosquitoes simultaneously silenced for *Caudal* (*Cad*) and immune effector gene *Rel1* (C)*, Rel2* (C)*,* or *Imd* (D). (E) *P. falciparum* sporozoite loads in the salivary glands of *An. gambiae* mosquitoes silenced for *Caudal* (*Cad*) or injected with dsRNA *GFP* (control). (F) *P. falciparum* oocyst loads in the midguts of *An. stephensi* non-transgenic (wt) and Cp transgenic mosquitoes silenced for *Caudal* (*Cad*) or injected with dsRNA *GFP* (control). For all figures, circles represent the number of parasites from individual mosquitoes, and horizontal lines represent the median number of parasites. Three biological replicates were performed, and indicated statistical significance was derived from Mann-Whitney tests. The *p*-values are presented where *: $p<0.05$ or $p<0.01$; **: $p<0.001$; ***: $p<0.0001$. Additional statistical analyses appear in Table S2.

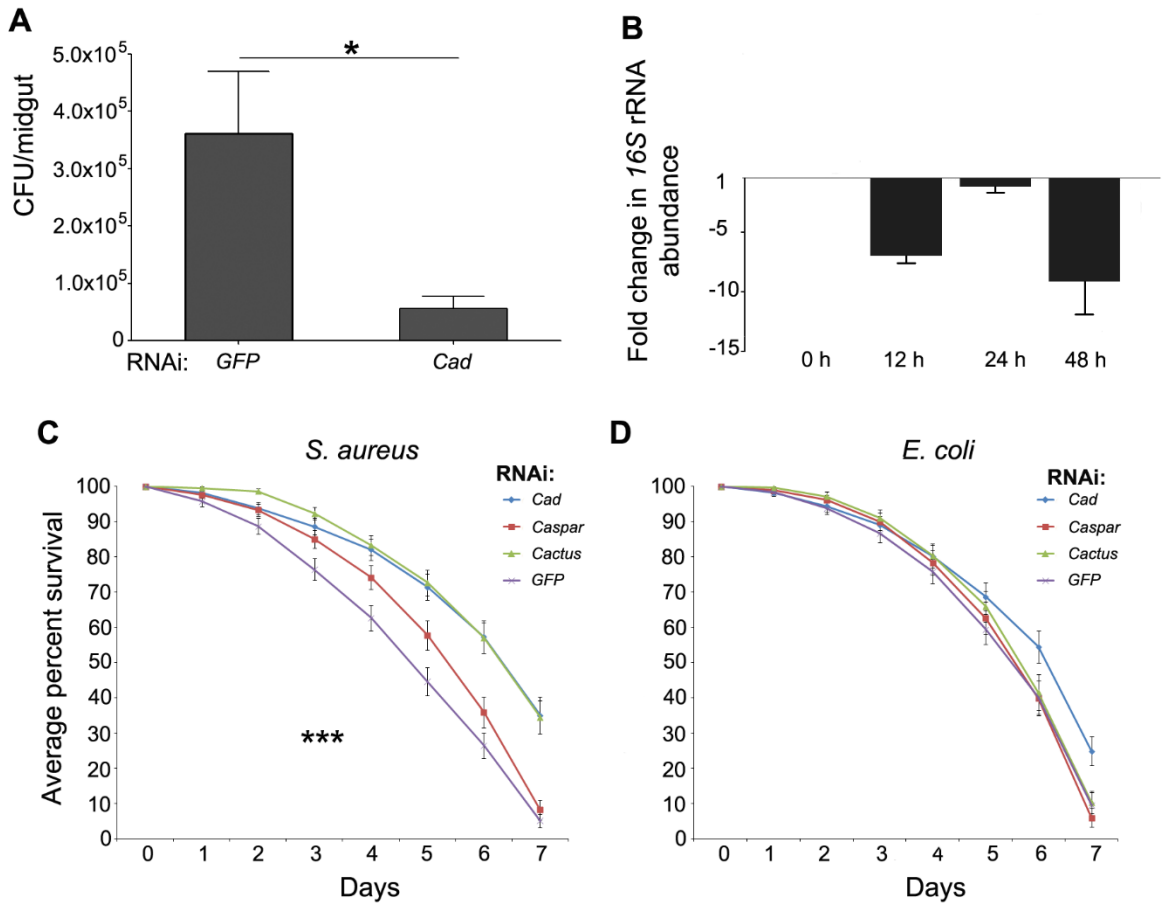


Fig. 2.3. Antibacterial responses in *Caudal*-silenced mosquitoes. (A) The midgut microbiota load was analyzed via colony-forming unit (CFU) assays in *An. gambiae Cad* and *GFP* dsRNA-treated mosquitoes. (B) Midgut-specific transcript abundance of microbial load (with the ribosomal gene *S7* used as an internal control) at 12, 24, and 48 h post RNAi-mediated silencing of *Cad* (the 0 h time point is also included). (C) Survival curves for *Cad*, *Cactus*, *Caspar*, and *GFP* dsRNA-treated mosquitoes systemically infected with *S. aureus* or (D) with *E. coli*. For both figures, survival was assessed for 7 days, and the curves indicate average percent survival. Statistical significance was observed for (C) but not for (D). For (A) and (B), columns and error bars represent mean

values and the standard error of the mean. Three biological replicates were conducted. The Mann-Whitney test was used for (A), and statistical significance is indicated where *: $p<0.05$ or $p<0.01$; **: $p<0.001$; ***: $p<0.0001$. For (C) and (D), Kaplan-Meier survival analysis was used with the log-rank (Mantel-Cox) test for significance evaluation. Supplementary information is provided in Table S3.

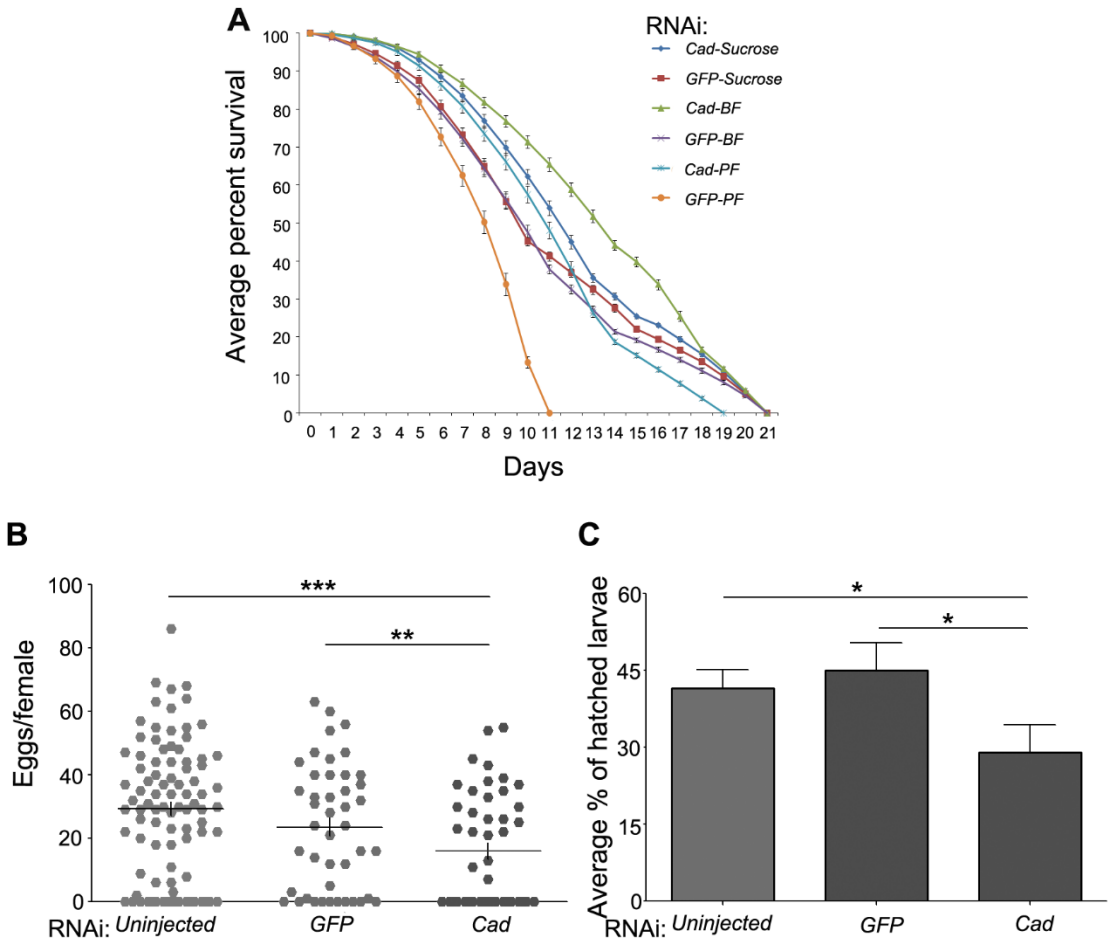


Fig. 2.4. Fitness outcomes in *Caudal*-silenced mosquitoes. (A) Longevity studies in *Cad* and *GFP* dsRNA-treated mosquitoes were conducted under the following conditions: (1) mosquitoes maintained on 10% sucrose solution; (2) mosquitoes given a single naïve blood meal and then maintained on 10% sucrose; or (3) mosquitoes given a

single *P. falciparum*-infected blood meal and then maintained on 10% sucrose. Survival was assessed until 100% mortality was reached. The curves indicate the average percent survival for each cohort. Kaplan-Meier survival analysis was used with the log-rank (Mantel-Cox) test for significance evaluation. Additional information is provided in Table S4. (B) Fecundity analysis in *Cad* and *GFP* dsRNA-treated and untreated mosquitoes; the circles represent the number of eggs laid per female after a single naïve blood meal; the horizontal bars represent the mean number of eggs laid per female, and error bars represent the standard error of the mean. Data were subjected to the Mann-Whitney statistical test, and the plots are data from three independent biological replicates. Statistical significance is indicated where *: $p<0.05$ or $p<0.01$; **: $p<0.001$; ***: $p<0.0001$. (C) Fertility analysis in *Cad* and *GFP* dsRNA-treated and untreated mosquitoes; hatch rates indicate the average percentage of eggs giving rise to first to second instar larvae. Error bars represent the standard error of the mean. The Mann-Whitney statistical test was used and significance is indicated where *: $p<0.05$ or $p<0.01$; **: $p<0.001$; ***: $p<0.0001$. Three biological replicates were conducted. See additional information in Table S5.

CHAPTER 3

CROSS-COLONIZATION AND CO-ADAPTATION CAPACITIES BETWEEN THE MIDGUT MICROBIOTA AND THE PHYLOGENETICALLY DISTINCT MOSQUITO SPECIES *ANOPHELES GAMBIAE* AND *AEDES AEGYPTI*

This work is currently being prepared as a manuscript:
Clayton, AM, Fastman, N, and Dimopoulos, G. **(2013)**. Cross-colonization and co-adaptation capacities between the midgut microbiota and the phylogenetically distinct mosquito species *Anopheles gambiae* and *Aedes aegypti*.

Abstract

Many studies have highlighted the importance of the mosquito midgut microbiota's role in the defense and inhibition of pathogens such as *Plasmodium* and dengue virus. The microbiota could potentially provide a novel and relatively low-tech/cost strategy to reduce disease transmission to humans. However, there is a lack of understanding of the mosquito-microbiota-pathogen interactions, and this needs to be undertaken before such strategies can be developed and implemented. In this pilot study, we used midgut bacterial isolates (from the genera *Escherichia*, *Acinetobacter*, *Asaia*, and *Pseudomonas*) derived from laboratory reared mosquitoes to investigate the cross-colonization and co-adaptation capacities between the midgut microbiota and the phylogenetically distinct mosquito species and important disease vectors *Aedes aegypti* and *Anopheles gambiae*. Our results highlighted the complexity that exists in multi-taxa host microbe interactions. Specifically, upon introducing single bacterial isolates, the majority of our cohort of bacterial isolates demonstrated the ability to cross-colonize the two mosquito hosts. However, the *Acinetobacter* strain derived from *An. gambiae* demonstrated co-species adaptation to the *An. gambiae* mosquito host rather than cross-colonization capacity. Interestingly, the *Asaia* strain derived from *Aedes* was able to colonize the *An. gambiae* host better than the *Aedes* mosquito host. Upon co-introducing our cohort of bacterial isolates in groups, we noticed that the bacterial isolates, regardless of which mosquito species they were derived from, were able to thrive and colonize in the *An. gambiae* midgut but not the *Aedes aegypti* midgut. Such observations have provided an impetus to investigate the role of the mosquito's innate immune system, specifically the anti-bacterial Imd immune pathway, in modulating cross-colonization

capacity between the midgut microbiota and mosquito hosts.

Keywords: *Anopheles, Aedes, midgut microbiota, co-adaptation, cross-colonization, innate immunity, mosquito.*

Introduction

There are more than 3,500 species of mosquitoes around the world [172]. The mosquito species *Anopheles gambiae* and *Aedes aegypti* have emerged as important vectors for the infectious diseases malaria and dengue, respectively. Over recent years, many studies have highlighted the contribution of midgut bacteria in influencing these mosquito vectors' competence to respectively defend against the malaria parasite *Plasmodium* and the flavivirus dengue [116,117,173,174]. Additionally, control strategies based on manipulating the mosquito host and midgut bacterial species may be a better avenue to pursue due to lack of effective vaccines and increased insecticide resistance in mosquitoes [1]. To demonstrate the practicality of using the midgut microbiota in disease control, a recent study has successfully demonstrated the use of genetically-modified midgut bacteria as a tool to combat malaria [122]. Thus, increasing knowledge of mosquito-microbiota interactions will subsequently allow for symbiont-based control strategies to be successfully implemented in the field.

The mosquito midgut is a key organ for nutrition and digestion, and displays a high level of immune-competence to ward off ingested pathogens and to control over-proliferation of the natural microbiota [175,176]. As earlier stated, a number of studies have highlighted the importance of active midgut bacterial species in immunity [117]. The composition of these microbial communities, or microbiota, is determined by the microbial exposure from the external environment as well as environmental factors

within the mosquito such as redox conditions, pH, digestive enzymes, the presence of other microorganisms, and the nutrient composition of food sources ingested [176,177]. Hence, the microbiota, in order to persist in its intimate relationship with the midgut, must be able to adapt to such an environment present within its mosquito host. Bacteria that emerge as successful in maintaining such a sustained relationship with the mosquito midgut are commonly referred as symbionts [178]. Mosquito-associated bacterial symbionts are typically defined as primary/obligate or secondary symbionts. Obligate, primary symbionts have co-evolved with the host and hence are essential to host survivability while secondary symbionts are facultative and may be recently acquired by the host. Secondary symbionts can contribute to host fitness and resistance and defense against pathogens [175]. Primary symbionts are usually vertically transmitted; vertical transmission may also occur in secondary symbionts, but secondary symbionts can colonize new hosts, within members of the same species or across different species via horizontal transmission. To date, there is no description of primary symbionts in mosquitoes [175]. Mosquito-microbiota studies have focused on secondary symbionts and their impact on various facets on mosquito host biology. Particularly, the acetic acid bacteria *Asaia* has emerged as an important symbiont of *Anopheles* and recent lines of evidence imply that *Asaia* establishment reduces *Plasmodium* infection [115,179-183].

Several studies have been conducted to assess the gut flora of a range of mosquito species from laboratory and field settings [116,173,174,184-188]. These studies, and others, have revealed that most mosquito midgut bacterial species mainly belong to the *Gammaproteobacteria* class [175,186,188,189]. Although, there is some information about the bacterial prevalence in mosquitoes, there is wide variation

in reported studies, with no one dominant species or genus characterized from the mosquito midgut. However, some of the most common bacterial genera isolated from mosquitoes are *Serratia*, *Klebsiella*, *Pseudomonas*, *Enterobacter*, *Escherichia*, *Acinetobacter*, *Proteus*, *Asaia* [183,189-191]. It has also been demonstrated that highly prevalent genera such as *Pantoea* and *Asaia* have the capacity to cross-colonize different mosquito species [180,181,185,192]. Such a capability further implies the versatility of secondary symbionts, particularly their ability to horizontally transmit and colonize. Such transmission routes and a wide host range are ideal qualities for the successful execution of symbiont-based strategies to control pathogen development and dissemination [175,180,183].

The purpose of this study was to carry out a comparative study of cross-colonization capacities of the midgut microbiota in the malaria vector *Anopheles gambiae* and the dengue vector *Aedes aegypti*. Our pilot study was conducted using laboratory reared mosquitoes and midgut microbiota derived from these mosquitoes. Similar studies have been conducted [180,185], however our study is unique in that we are not only simultaneously looking at microbiota cross-colonization capacities across these two mosquito species, but this study will be followed up with an investigation of how the Imd (Immune deficiency) immune pathway may modulate such mosquito-microbiota interactions. As earlier discussed, midgut symbionts play an active role in immunity. Particularly, the midgut microbiota is kept in check by the innate immune system to maintain a balanced microbial flora [22]. The commensal bacteria stimulate basal innate immune activity consisting of the induction of antimicrobial peptides (AMPs) and other immune effectors that act against *Plasmodium* or dengue [114,117]. The Imd pathway

has been shown to play an active role in this process as a key regulator of immune responses in the *Drosophila* and mosquito gut [113,154]. Studies done in *Drosophila* and *Anopheles* have particularly highlighted that the transcription factor and a negative regulator of the Imd pathway, Caudal, regulates the proliferation and species composition of the insect midgut microbiota and influences AMP transcript abundance [41,42]. In a follow up study, there will be an assessment of the Imd pathway's influence on the midgut microbiota's cross-colonization capacity by silencing the *Imd* and *Caudal* genes, via RNAi, in *Ae. aegypti* and *An. gambiae* and then introduce our cohort of bacterial isolates.

Methods and Materials

Mosquito rearing and mosquito strains

An. gambiae Keele and *Ae. aegypti* Rockefeller strain mosquitoes were maintained under laboratory conditions at 27°C and 80% humidity with a 12 h day-night cycle. Larvae were reared on cat food pellets and ground fish food supplement. Adult mosquitoes were maintained on 10% sucrose and fed on blood for egg production [145,173].

Characterization and isolation of proliferated midgut microbial flora

Isolation and colony-forming unit (CFU) enumeration of endogenous gut bacteria from 2-3 d old wildtype mosquitoes were performed as previously described [114]. Briefly, mosquitoes were surfaced-sterilized by dipping them in 100-70% EtOH for 2 min and then rinsing them with 1X PBS twice for 1 min each. Midguts were dissected from each individual mosquito over a sterile glass slide containing a drop of 1X PBS, then transferred to a microcentrifuge tube containing 150 ul of sterile 1X PBS, and

macerated for 30 sec. Three 10-fold serial dilutions were then plated on LB agar and kept at room temperature for 48 h. Initial isolation was based on morphology, color, and size of the colony and was followed by molecular identification via *16S* rDNA sequencing. Primers used to amplify the *16S* rDNA were: Forward, AGAGTTTGATCCTGGCTCAG; Reverse (degenerate), TACGGYTACGCTTGTTACGACT [116]. Selected bacterial isolates, and designated names, used in this study are listed in Supplementary Tables S1 and S2. We selected *Acinetobacter* and *Pseudomonas* strains from *Anopheles gambiae*, and *Pseudomonas*, *Escherichia*, and *Asaia* strains from laboratory *Aedes aegypti*. We selected bacterial isolates from genera that have been previously identified as common secondary symbionts identified in mosquitoes (see Introduction). *16S* rRNA-specific primers were made for selected bacterial isolates (see Table S3) in order to use for qRT-PCR analysis.

In-vitro growth dynamics, bacterial interspecies growth inhibition, and antibiotic resistance assays

After selecting our 5 bacterial isolates, we assessed if the respective 2 *Anopheles*-derived bacterial isolates and 3 *Aedes*-derived bacterial isolates exhibited any growth inhibition abilities by conducting disk inhibition assays according to standard protocols [193-195]. Briefly, 200 ul of an overnight (15h) culture of each bacterial isolate was plated on LB agar. Then, filter paper disks containing another bacterial isolate was placed upon the LB agar plate. Zones of inhibition (ZOI) were then measured in mm 1-2 days later. Three replicates were conducted. This information is provided in Tables S4 and S5. None of our isolates displayed growth inhibition.

In this study, we re-introduced bacterial isolates via a blood meal. To ensure that

our mosquito-derived isolates could replicate in a microenvironment composed of blood, we grew our isolates overnight (15h) in a 50% LB: 50% blood liquid media. As controls, we similarly grew our isolates in LB liquid media. We then conducted CFU assays using LB agar plates. Three replicates were conducted. This data is provided in Supplementary Figure S1. One-way ANOVA with a Tukey's multiple comparison test was used to determine if bacterial growth was significantly influenced by the presence of blood. We noticed that at least *in-vitro*, all of our isolates were able to replicate in the presence of blood.

Prior to introducing the bacterial isolates, we antibiotically treated mosquitoes using a Penicillin/Streptomycin regimen in order to diminish the presence of their midgut microbiota. To address if any residual antibiotics, perhaps not eliminated by supplying mosquitoes with sterile water before blood feeding, would affect colonization capacities in our studies, we conducted *in-vitro* antibiotic resistance assays with our bacterial isolates using standard disk inhibition assay protocols [193-195]. Briefly, we plated 200 ul of an overnight culture on a LB agar plate. Then, disks containing either 10,000, 100, 10, or 1 ug/mL of Penicillin/Streptomycin (PS or PenStrep) were placed on the plate. ZOIs were then measured 1-2 days later. Three replicates were conducted. This data is provided in Table S6. *In-vitro*, our bacterial isolates are able to grow in the presence of dilute antibiotics.

Mosquito antibiotic treatment and reintroduction of selected bacterial isolates through blood meal

Mosquitoes were rendered aseptic by maintaining them on a 10% sucrose solution with 20 units penicillin and 20 ug streptomycin (PS or PenStrep) since the first day post-

eclosion until two days prior to challenge [173]. Mosquitoes were then maintained for 1 day on sterile water and starved for 6-10 hours prior to blood meal. Effectiveness of antimicrobial treatment was confirmed by CFU assays and/or *16S* rRNA qRT-PCR analysis (using universal *16S* rRNA primers, see Table S3) prior to blood feeding and bacterial challenge (data not shown).

Mosquitoes were fed on a mix containing 50% bacteria suspended in 1X PBS (final concentration: 10^3 bacteria/mL; for controls, only 1X PBS was added), 20% of human commercial blood, and 30% human serum for 45 minutes. To be clear, we fed mosquitoes with each isolate (10^3 bacteria/mL) respectfully derived from either *Anopheles* or *Aedes* mosquitoes. We also conducted experiments in which we fed mosquitoes a cohort of the bacterial isolates (at a final concentration of 10^3 bacteria/mL) derived from *Anopheles* or *Aedes* mosquitoes. We also had control mosquitoes that were given a naïve blood meal (supplemented with 1X PBS). A visual representation of our feeding schemes is provided in Supplementary Figures S2 and S3. Mosquitoes which did not feed were removed. After blood meal, all cohorts were maintained on sterile 1% sucrose solution, a methodology based on previous work [173]. Following the bacterial reintroduction via blood meal, midguts were dissected at 12 hours, 2 days (48 h), 4 days (96 h), and 6 days (144 h) in order to conduct *16S* rRNA qRT-PCR analysis. Three replicates were conducted.

RNA isolation, quantitative real-time PCR (qRT-PCR), and RNA interference (RNAi)-mediated gene silencing

RNA was extracted and bacterium-specific *16S* rRNA abundance was quantified

(in triplicate samples) in the midgut tissue using the RNeasy kit (Qiagen). cDNA was prepared using 50:50 mix of oligo(dT_{20}) and random hexamers primers and the Invitrogen Superscript III reverse transcriptase according to standard methodology. The quantitative real-time PCR (qRT-PCR) and RNAi gene-silencing assays were carried out according to [34], and the ribosomal protein S7 gene was used for normalization of the cDNA templates. The fold change in the gene expression and the gene silencing efficiency (from the RNAi assays) were calculated according to the standard EΔΔCt method [146] when both primer efficiencies of the GOI (gene of interest) and the *S7* gene were equal. The primer efficiencies were determined as described in [146].

An. gambiae and *Ae. aegypti* immune genes *IMD* and *Caudal* will be screened for their potential role in modulating mosquito-microbiota co-adaptation and/or microbiota cross-colonization capacities using RNA interference (RNAi) in wildtype mosquitoes. For these assays, the mRNA for the specific gene will be selectively depleted from the adult female mosquitoes using established RNAi methodology [147]. The dsRNA injection assay of different genes will be repeated three times with at least 50-80 mosquitoes in each experiment; the GFP dsRNA-injected mosquitoes will serve as controls. The RNAi gene silencing efficiencies will be determined at 2-3 d post dsRNA injection for all genes tested and compared to the GFP dsRNA-injected control mosquitoes. The primers used for dsRNA synthesis and silencing verification are presented in Table S3.

Statistical analysis

Real-time PCR assays were normalized and standardized according to [196]. One-way ANOVA with a Tukey's multiple comparison test was used when appropriate.

Statistical analyses were conducted using GraphPad Prism statistical software package (GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com"). Statistical significance is indicated with asterisks: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

Results and Discussion

Properties of selected bacterial isolates

As mentioned in the Methods and Materials, we selected *Acinetobacter* and *Pseudomonas* strains from *Anopheles gambiae*, and *Pseudomonas*, *Escherichia*, and *Asaia* strains from *Aedes aegypti* for this study based on morphological discrimination of colonies, and because (See Supplementary Tables S1 and S2) these isolates represent genera that have been previously identified as common secondary symbionts in mosquitoes (see Introduction).

We first assessed whether the respective two *Anopheles*-derived bacterial isolates and three *Aedes*-derived bacterial isolates exhibited any growth inhibition properties to any of the isolates by conducting disk inhibition assays according to standard protocols (see Methods and Materials, and Tables S4 and S5). None of our isolates exhibited growth inhibition properties *in vitro*.

In this study, we re-introduced bacterial isolates into the mosquito midgut via a blood meal. This method of administration was chosen since there is better control of how much bacteria is imbibed by each mosquito and also ease of selecting fed versus non-fed mosquitoes as compared to sugar-feeding methodology. Also, understanding the dynamics of our selected bacterial isolates in the context of blood may provide useful knowledge of their potential role in shaping the microbial community and midgut

environment in the context of blood borne pathogenic infections (such as *Plasmodium* or dengue virus infection). Previous studies have shown that the blood fed midgut is a highly reducing environment, and the structure of the microbial community is changed drastically, favoring enteric bacteria and bacteria possessing hemolytic activity [177,197,198]. Recent genomic analyses demonstrate that the enteric bacteria have a large redox capacity to handle the oxidative, nitrosative stresses associated with the breakdown of the blood meal, suggesting a beneficial role in maintaining gut redox homeostasis [197]. The midgut bacterial redox capacity could also influence the production of anti-*Plasmodium* nitrogen oxides [75,198].

To ensure that our mosquito-derived isolates could replicate in a microenvironment composed of blood, we cultured our isolates overnight in a blood-media mix (see Methods and Materials). We noticed that at least *in-vitro*, all of our isolates were able to replicate in the presence of blood (Supplementary Figure S1). Additionally, a blood microenvironment did not significantly enhance or inhibit growth of our bacterial isolates.

Prior to introducing the bacterial isolates, we treated mosquitoes using a Penicillin/Streptomycin regimen in order to diminish the presence of their midgut microbiota (see Methods and Materials). To address if any residual antibiotics would affect colonization capacities in our studies, we conducted *in-vitro* antibiotic resistance assays with our bacterial isolates using standard disk inhibition assay protocols (see Methods and Materials). *In-vitro*, our bacterial isolates are able to grow in the presence of dilute antibiotics (Supplementary Table S6).

Dynamics of microbiota colonization of the Aedes* and *Anopheles mosquito midgut

In this study, we assessed cross-colonization capacities of our selected isolates in laboratory-reared *Aedes* and *Anopheles* mosquitoes. We introduced our isolates either individually or together in a cocktail via a blood meal and then assessed their abundance by measuring their *16S* rRNA levels 12, 48, 96, and 144 h post-blood meal. The cocktail (grouped) bacterial feedings comprised of isolates derived from *Aedes* (*AeL*) or *Anopheles* (*AgL*) mosquito hosts. Please refer to Supplementary Figures S2 and S3 for our feeding schemes.

We first assessed colonization dynamics of our singly introduced *Aedes*-derived bacterial isolates *Esc_sp_Ae*, *Asa_sp_Ae*, and *Pse_sp_Ae* in our two mosquito host species (Fig. 3.1A-C). Our *Esc_sp_Ae* strain was able to effectively colonize both mosquito species, hence indicating this strain's capability to cross-colonize (Fig. 3.1A). Similarly, our *Pse_sp_Ae* strain also demonstrated cross-colonization capacity amongst our laboratory reared mosquito hosts (Fig. 3.1C). Interestingly, our *Asa_sp_Ae* strain did not appear to colonize the midgut of either mosquito species very well based on *16S* rRNA transcript abundance (Fig. 3.1B). However, this strain was able to better colonize the *Anopheles gambiae* than the *Aedes aegypti* midgut, the original host from which it was derived. We note this as an interesting finding as it may imply that the *An. gambiae* midgut may provide a more favorable microenvironment than the *Aedes aegypti* midgut for this strain of *Asaia*. Alternatively, the antibacterial immune response elicited upon introduction of this bacterial strain is possibly higher in *Aedes aegypti* than in *Anopheles gambiae*. A recent study suggests that *Aedes* mosquitoes induce higher levels of AMP transcripts compared to *Anopheles* upon bacterial infection [156]. We also note that previous studies investigating the cross-colonization capacity of *Asaia* suggests that

Asaia is a symbiont that possesses the ability to establish itself in the guts of different mosquito and other insect species [180,181]. Our data suggests that perhaps certain strains of *Asaia* may have such a broad host spectrum, but our strain is different from the *Asaia* strain used in previous studies [115,180,199]. We also note that we cannot exclude the possibility that the mosquito-microbiota dynamics that have been observed thus far are mosquito strain specific. Hence, our observations may not necessarily be generalized to represent all strains of *An. gambiae* and *Ae. aegypti*.

We next assessed cross-colonization capacity of our singly introduced *Anopheles*-derived bacterial isolates *Pse_sp_Ag* and *Aci_sp_Ag* (Fig. 3.2A-B). Our data suggests that the *Pse_sp_Ag* strain is capable of cross-colonizing both mosquito hosts efficiently (Fig. 3.2A). However, our *Aci_sp_Ag* strain demonstrates co-species adaptation and appears to poorly colonize *Aedes* mosquitoes (Fig. 3.2B). This observation indicate that the *An. gambiae* midgut likely possesses a microenvironment favorable for the growth of this *Acinetobacter* strain. Alternatively and as previously discussed, perhaps in the *Aedes*' midgut, there are increased transcriptional activation of AMPs that compromise bacterial colonization [85].

Our *in-vivo* assessment of mosquito-microbiota interactions suggest that some bacterial isolates display a co-adaptation relationship with a particular mosquito host whereas other bacterial isolates display broad mosquito host range and can colonize both species efficiently. Also in our studies, when we introduced a cocktail of either *Aedes*-derived (*AeL*) or *Anopheles*-derived (*AgL*) bacterial isolates, none of these isolates could successfully colonize in the *Aedes* gut (Fig. 3.3A-B). This could be indicative of a pronounced elicitation of AMPs as earlier discussed. Alternatively, it could imply that

interspecies competition is more pronounced in the *Aedes aegypti* midgut microenvironment compared to perhaps a more permissive *An. gambiae* midgut. As earlier mentioned, we note that these observations may be mosquito strain specific and not be inclusive to other strains of *An. gambiae* and *Ae. aegypti*.

It will be interesting and fruitful to investigate the role of the Imd pathway in modulating the cross-colonization capacity of the midgut microbiota. Particularly, it would be interesting to investigate the role of the midgut microbial load regulator and Imd negative regulator Caudal in mosquito-microbiota dynamics. Studies done in the fruit fly and *An. gambiae* indicates that this factor modulates bacterial load and species composition [41,42].

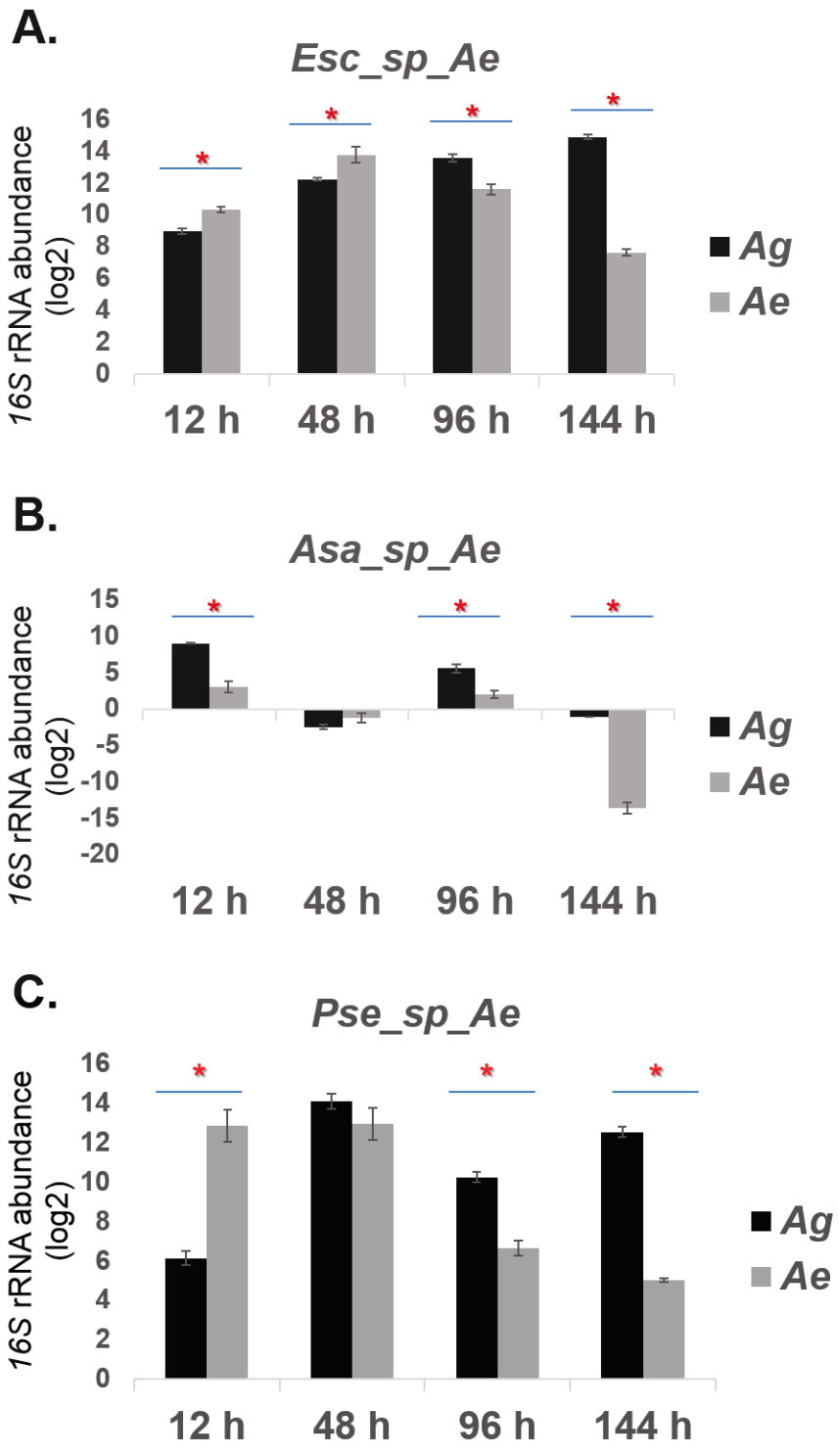


Fig. 3.1. *16S* rRNA abundance of *Aedes*-derived bacterial isolates in *An. gambiae* and *Ae. aegypti* wildtype mosquitoes (single infections). *16S* rRNA transcript

abundance of *Esc_sp_Ae* (A), *Asa_sp_Ae* (B), and *Pse_sp_Ae* (C) in the midguts of wildtype *Anopheles* (*Ag*) and *Aedes* (*Ae*) female mosquitoes (ribosomal gene *S7* used as an internal control) at 12, 48, 96, and 144 h post bacteria-blood meal. To clarify, this data represents the bacteria-specific *16S* rRNA expression relative to the bacteria-specific *16S* rRNA expression found in antibiotic treated mosquito midguts given a naïve blood meal and then maintained on sterile 1% sucrose solution. Each column and error bar respectively represents the transcript abundance as log2 values and the standard error of the mean. Data were subjected to the One-way ANOVA with a Tukey's multiple comparison statistical test. Statistical significance is indicated where *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. For all figures, three replicates were performed.

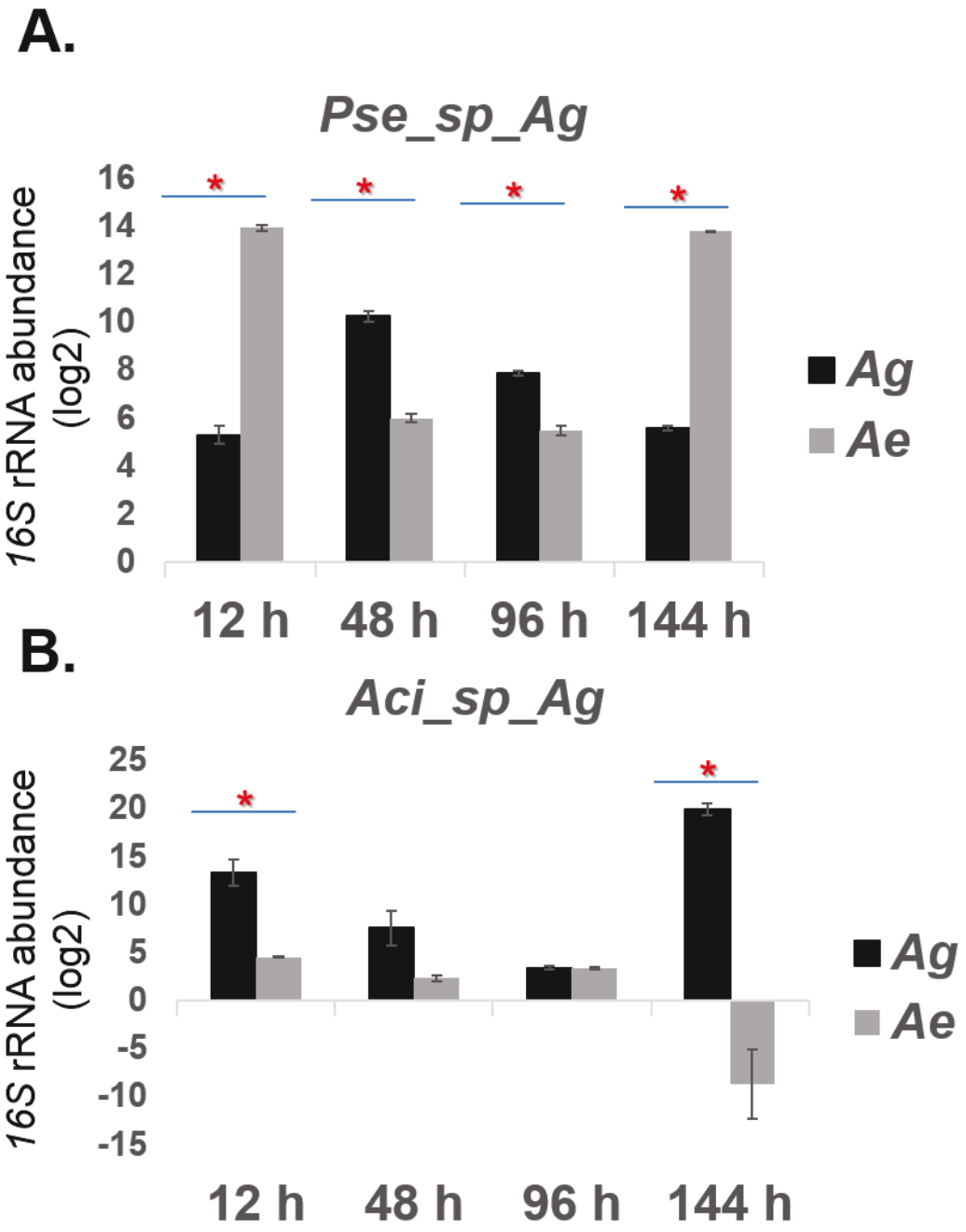


Fig. 3.2. *16S* rRNA abundance of *Anopheles*-derived bacterial isolates in *An. gambiae* and *Ae. aegypti* wildtype mosquitoes (single infections). *16S* rRNA transcript abundance of *Pse_sp_Ag* (A), *Aci_sp_Ag* (B) in the midguts of wildtype *Anopheles* (*Ag*) and *Aedes* (*Ae*) female mosquitoes (ribosomal gene *S7* used as an internal control) at 12,

48, 96, and 144 h post bacteria-blood meal. To clarify, this data represents the bacteria-specific *16S* rRNA expression relative to the bacteria-specific *16S* rRNA expression found in antibiotic treated mosquito midguts given a naïve blood meal and then maintained on sterile 1% sucrose solution. Each column and error bar respectively represents the transcript abundance as log2 values and the standard error of the mean. Data were subjected to the One-way ANOVA with a Tukey's multiple comparison statistical test. Statistical significance is indicated where *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. For all figures, three replicates were performed.

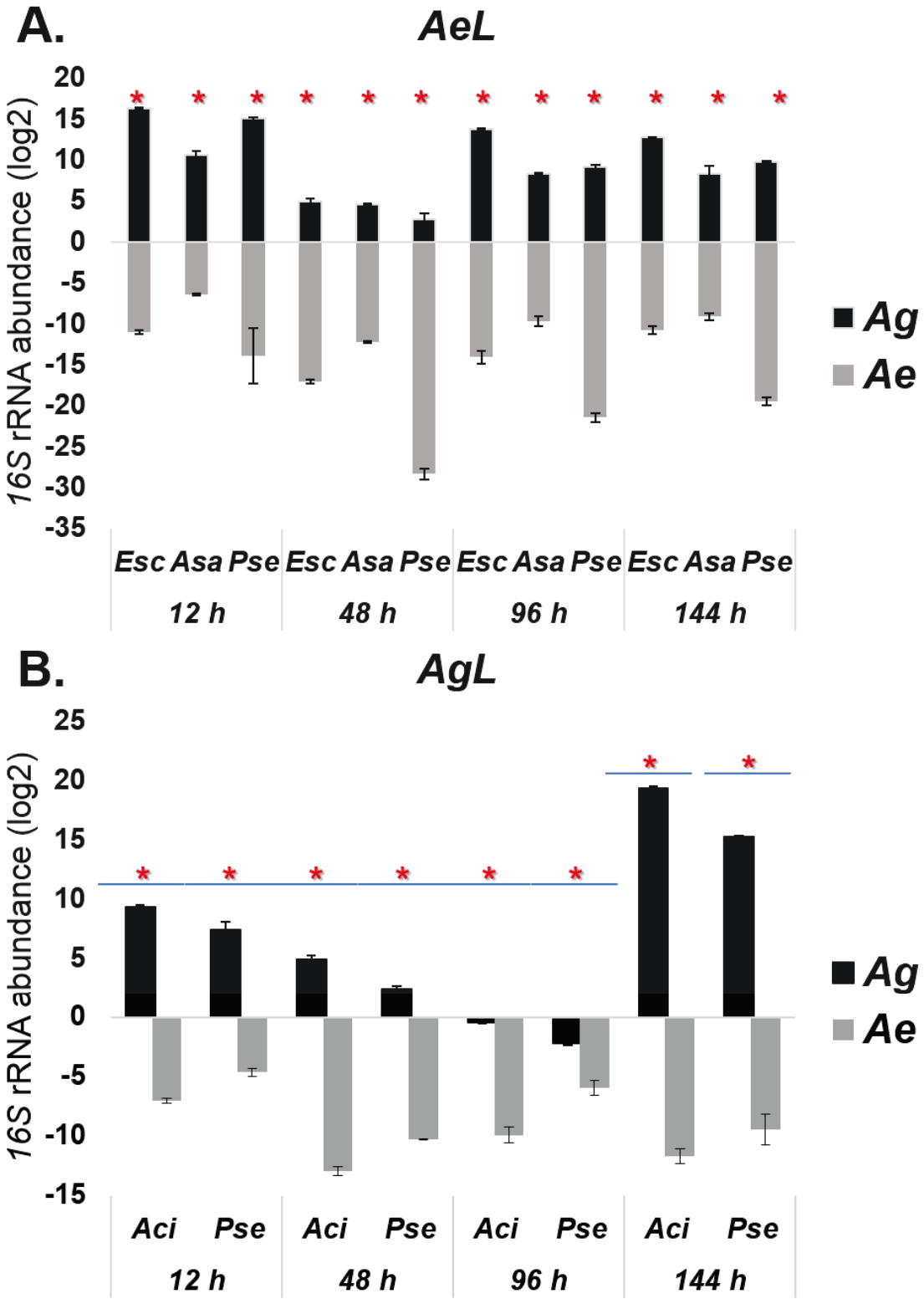


Fig. 3.3. ***16S*** **rRNA abundance of** ***Anopheles*****-derived and** ***Aedes*****-bacterial isolates in**

***An. gambiae* and *Ae. aegypti* wildtype mosquitoes (group infections).** *16S* rRNA transcript abundance of *Esc_sp_Ae*, *Asa_sp_Ae*, and *Pse_sp_Ae* (A; *AeL*) and *Pse_sp_Ag* and *Aci_sp_Ag* (B; *AgL*) in the midguts of wildtype *Anopheles* (*Ag*) and *Aedes* (*Ae*) female mosquitoes (ribosomal gene *S7* used as an internal control) at 12, 48, 96, and 144 h post bacteria-blood meal. To clarify, this data represents the bacteria-specific *16S* rRNA expression relative to the bacteria-specific *16S* rRNA expression found in antibiotic treated mosquito midguts given a naïve blood meal and then maintained on sterile 1% sucrose solution. Each column and error bar respectively represents the transcript abundance as log2 values and the standard error of the mean. Data were subjected to the One-way ANOVA with a Tukey's multiple comparison statistical test. Statistical significance is indicated where *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. For all figures, three replicates were performed.

CHAPTER 4
DISCUSSION

Malaria, caused by the *Plasmodium* parasite, is a huge public health threat, affecting approximately 3 billion people worldwide each year. The major vector for *P. falciparum* in sub-Saharan Africa is the female *Anopheles gambiae* mosquito. Given the lack of an effective vaccine against *Plasmodium* and the increased resistance of this parasite to the current arsenal of drugs and of *Anopheles* mosquitoes to insecticides, the development of novel control strategies is crucial to reducing malaria transmission [1]. Studies exploring the mosquito's innate immune defense against *Plasmodium* may contribute towards the development of such preventive and control strategies. Additionally, many studies have highlighted the importance of the mosquito midgut microbiota's role in the defense and inhibition of pathogens, like *Plasmodium* and the dengue virus. The microbiota potentially provides a novel and relatively low-tech strategy to reduce disease transmission to man.

This doctoral research thesis specifically aimed to investigate immune factors that influence defense against the malaria parasite in the *Anopheles* mosquito. Additionally, the research discussed in this thesis investigated mosquito-microbiota interactions. In Chapter 2, we show that in *Anopheles gambiae,* the midgut-specific transcription immune factor *Caudal* acts as a negative regulator in the Imd pathway-mediated immune response against the human malaria parasite *P. falciparum*. *Caudal* also modulates the mosquito midgut bacterial flora. RNAi-mediated silencing of *Caudal* enhanced the mosquito's resistance to bacterial infections and increased the transcriptional abundance of key immune effector genes. Interestingly, *Caudal*'s silencing resulted in an increased lifespan of the mosquito, while it impaired reproductive fitness with respect to egg laying and hatching.

In Chapter 3, our *in-vivo* assessment of mosquito-microbiota interactions suggest that some bacterial isolates, *Aci_sp_Ag*, display a co-adaptation relationship with a particular mosquito host whereas other bacterial isolates, *Esc_sp_Ae*, *Pse_sp_Ag*, and *Pse_sp_Ae*, display broad mosquito host range and can cross-colonize efficiently. Interestingly, our *Asa_sp_Ae* strain that was originally derived from *Aedes aegypti* was able to establish itself better in *Anopheles gambiae* mosquitoes. Also in our studies, when we introduced a cocktail of either *Aedes*-derived (*AeL*) or *Anopheles*-derived (*AgL*) bacterial isolates, none of these isolates could successfully colonize in the *Aedes* gut. This could be indicative of a pronounced elicitation of AMPs and corroborate recent studies [85]. Alternatively, it could imply interspecies competition is more pronounced in the *Aedes* midgut microenvironment compared to perhaps a more permissive *An. gambiae* midgut.

Overall, the results discussed in Chapter 3 highlight the complexity that exists in multi-taxa host pathogen interactions. Additionally, it will be interesting to investigate the role of the Imd immune pathway in modulating the cross-colonization capacity of the midgut microbiota. Particularly, we would like to investigate the role of the midgut microbial load regulator and Imd negative regulator Caudal in mosquito-microbiota dynamics. Studies done in the fruit fly and *An. gambiae* (see Chapter 2) indicates that this factor modulates bacterial load and species composition [41,42].

Our studies presented in this thesis shed light upon the essential contribution of mosquito immune defenses to malaria infection. This thesis research also illuminates the importance of the interactions between the midgut microbiota, innate immune system, and the mosquito host. Lastly, our studies have also provided knowledge about mosquito-microbiota dynamics that may be useful for future detailed studies purposed to implement

microbiota-based control strategies in the field.

APPENDIX A: SUPPLEMENTARY DATA

Table S1. Primers used for gene expression analysis, dsRNA synthesis, and qRT-PCR validation of RNAi-mediated gene silencing and the efficiencies of gene silencing. Underlined letters indicate the T7 promoter sequence of the forward (RNAiF) and reverse RNAi primers (RNAiR) used for dsRNA synthesis. For gene silencing validation, KD% (± SEM) denotes the mean percent efficiency of gene knock-down (KD %) with standard error of the mean (SEM). The silencing efficiency was determined at 2-3 d post dsRNA injection. It is important to note that the table below denotes silencing efficiency in the midguts of dsRNA-injected mosquitoes.

Gene Name	Primer Name	Primer sequence (5'-3')	KD% (± SEM)
AgS7	AgS7-F	CCATCCTGGAGGATCTGGTA	
	AgS7-R	GATGGTGGTCTGCTGGTTCT	
AgCec1	AgCec1-F	CCAGAGACCAACCAACCACCAA	
	AgCec1-R	GCACTGCCAGCACGACAAAGA	
AgCec3	AgCec3-F	GTGCGCCGCGGTGGAAGT	
	AgCec3-R	AATGACGGGCAGCGCTTTCTTAG	
AgGam	AgGam-F	GTTTGCTTACGCGCCGACTTGT	
	AgGam-R	AAACGCCCTTCCGGTTGAGATAG	
AgDef1	AgDef1-F	CATGCCGCGCTGGAGAACTA	
	AgDef1-R	GATAGCGGCGAGCGATACAGTGA	
AgLRRD7	AgLRRD7-F	TCGGTGAGCAACAGTTTGAC	
	AgLRRD7-R	CAGGTCGAGATGGGTGAACT	
AgFBN9	AgFBN9-F	TTGTGATGAAGGAGCACAGC	

	AgFBN9-R	GCTTGATCCAACCGACTGAT	
AgCad	AgCad-F	ACCAGAGTCAACCCAATCCA	
	AgCad-R	ATCGTGATGTAGCGCGTGTA	
AgCactus	AgCactus-F	GAACGTTTCGACCGTTTGAT	
	AgCactus-R	TCAGAAACTGCTGTGGAACG	
AgCaspar	AgCaspar-F	CTCCCTTCATCGAACTCTGC	
	AgCaspar-R	ATGGTGCTGCTCACACACTC	
AgIMD	AgIMD-F	CGAAGCTAGAGACCGATGCT	
	AgIMD-R	ATTCCCATTTTGCGTAGCAG	
AgRel1	AgRel1-F	TAGCCCGTAAGCATCCATTC	
	AgRel1-R	TGCCAATGGTCTGTTGGTAA	
AsCad	AsCad-F	GCATCAGATGAGTGCGATGA	
	AsCad-R	TCACATTATGCTGAGCGATG	
AsS7	AsS7-F	TCGGTTCCAAGGTGATCAAAGC	
	AsS7-R	AGCGCGGTCTCTTCTGCTTGT	
AgRel2	AgRel2-F	GGTGGTGGTGTTATCGGTTC	
	AgRel2-R	CGACGTTGGTTCTTGACCTT	
16S rRNA	16s-F	TCCTACGGGAGGCAGCAGT	
	16s-R	GGACTACCAGGGTATCTAATCCTGTT	
GFP	GFP-RNAiF	TAATACGACTCACTATAGGATGGTGAG CAAGGGCGAGGAGCTGT	
	GFP-RNAiR	TAATACGACTCACTATAGGTTACTTGTA	

		CAGCTCGTCCATGCCG	
AgCad	AgCad-T7F	TAATACGACTCACTATAGGGCCATGTACTATCCCCATCCG	65.23% (± 0.95)
	AgCad-T7R	TAATACGACTCACTATAGGGTGTCTTTCGTGCGTGTCTTC	
AgCactus	AgCactus-T7F	TAATACGACTCACTATAGTAACACTGCGCTTCATTTGG	58.41% (± 0.90)
	AgCactus-T7R	TAATACGACTCACTATAGGCCCTTTTCAATGCTGATGT	
AgCaspar	AgCaspar-T7F	TAATACGACTCACTATAGCCGCTTTTCTAAACGCTGTC	42.61% (± 0.26)
	AgCaspar-T7R	TAATACGACTCACTATAGAAACAGGTTGCATGTGTGGA	
AsCad	AsCad-T7F	TAATACGACTCACTATAGAAGACAAGTACCGGGTGGTG	76.03% (± 0.58)
	AsCad-T7R	TAATACGACTCACTATAGGAGGTCGGAACACCGAGAT	
AgIMD	AgIMD-T7F	TAATACGACTCACTATAGGAATTTCCCAAATGGTGTG	44.74% (± 0.44)
	AgIMD-T7R	TAATACGACTCACTATAGTGTGTAGATTGCTCGCGTTC	
AgRel1	AgRel1-T7F	TAATACGACTCACTATAGATCAACAGC	53.20% (± 0.77)

		ACGACGATGAG	
	AgRel1-T7R	TAATACGACTCACTATAGTCGAAAAAG CGCACCTTAAT	
AgRel2	AgRel2-T7F	TAATACGACTCACTATAGCGGAGAAGT CGAAGAAAACG	42.59% (± 0.40)
	AgRel2-T7R	TAATACGACTCACTATAGCACAGGCAC ACCTGATTGAG	

Table S2. Statistical analyses of oocyst or sporozoite loads in dsRNA-treated mosquito midguts or salivary glands, respectively.

n: total midguts or salivary glands numbers; range: range of oocysts or sporozoites numbers from 3 biological replicates; prevalence: % of mosquitoes with at least one parasite; median (with zeros): median oocysts or sporozoites from 3 biological replicates; mean (with zeros): mean oocysts or sporozoites from 3 biological replicates. The *p*-values from Mann-Whitney test are presented where *: $p<0.05$ or $p<0.01$; **: $p<0.001$; ***: $p<0.0001$; ns: no significance. Further statistical analyses were performed as needed using the Kruskal-Wallis test with a Dunn's multiple comparison post-test.

Fig. 2A (Pf oocysts)(0.1-0.5% gametocytemia)	***dsGFP***	***dsCad***
n=	56	51
Range	0-173	0-96
Prevalence	85.7%	72.5%
Median (with zeros)	18.5	6.0
Mean (with zeros)	32.3	14.5
Mann-Whitney test *p*-value	*;0.0125	
Fig. 2B (Pb oocysts)	***dsGFP***	***dsCad***
n=	78	68
Range	0-222	0-198
Prevalence	85.9%	88.2%
Median (with zeros)	28.5	18
Mean (with zeros)	39.2	33.8
Mann-Whitney test *p*-value	ns;0.6847	

Fig. 2C (Pf oocysts) (0.05-0.1% gametocytemia)	***dsGFP***	***dsCad***	***dsCad/Rel1***	***dsCad/Rel2***
n=	59	36	43	36
Range	0-60	0-49	0-35	0-88

Prevalence	91.5%	83.3%	86.0%	86.1%
Median (with zeros)	22	9.5	9.0	18
Mean (with zeros)	21.3	10.6	10.8	26.0
Kruskal-Wallis test	***	***	***	***
Dunn's multiple comparison post-test		**	**	ns
Mann-Whitney test *p*-value		***;0.0002	***;0.0003	ns;0.8029
Fig. 2D (Pf oocysts) (0.01-0.05% gametocytemia)	***dsGFP***	***dsCad***	***dsCaspar***	***dsCad/IMD***
n=	79	43	41	40
Range	0-24	0-10	0-13	0-15
Prevalence	94.9%	81.4%	70.7%	85.0%
Median (with zeros)	6	5	4	6
Mean (with zeros)	7.5	4.3	4.2	5.9
Kruskal-Wallis test	**	**	**	**
Dunn's multiple comparison summary		*	**	ns
Mann-Whitney test *p*-value		**;0.0031	***;0.0009	ns;0.2567

Fig. 2E (Pf sporozoites) (0.01-0.05% gametocytemia)	***dsGFP***	***dsCad***
n=	39	50
Range	75-2100	0-1350
Prevalence	100.0%	92.0%
Median (with zeros)	975	300
Mean (with zeros)	1019	409.5
Mann-Whitney test *p*-value	***; <0.0001	

An. stephensi transgenic mosquitoes				
Fig. 2F (Pf oocysts) (0.01-0.05% gametocytemia)	***WT***		***Cp***	
	dsGFP	***dsCad***	***dsGFP***	***dsCad***

n=	50	50	32	30
Range	0-10	0-8	0-8	0-5
Prevalence	66%	46%	50%	26.7%
Median (with zeros)	2	0	0.5	0
Mean (with zeros)	2.8	1.0	1.6	0.6
Mann-Whitney test *p*-value		**;0.0028		*; 0.0362

Table S3. Kaplan-Meier survival analyses of dsRNA-treated mosquitoes after systemic bacterial infections.

dsRNA-treated mosquitoes were either injected with *E. coli* (OD_{600} 3.5), *S. aureus* (OD_{600} 0.8), or 1X PBS. Survival was assessed for seven days post infection with bacteria or 1X PBS. Three independent experiments were performed, and the data are provided below for each experiment.

Treatment: 1X PBS				
Av. number of mosquitoes per group: 25				
Experiment 1	*Percent Survival*			
Days	*dsCad*	*dsCaspar*	*dsCactus*	*dsGFP*
0	*100*	*100*	*100*	*100*
1	*100*	*99.52381*	*99.52381*	*98.21429*
2	*100*	*98.9709*	*97.86508*	*95.48611*
3	*100*	*98.3111*	*95.90778*	*92.30324*
4	*100*	*96.67258*	*91.11239*	*88.45728*
5	*100*	*91.30188*	*84.02587*	*82.31441*
6	*98.33334*	*80.64999*	*71.42199*	*73.73999*
7	*72.11111*	*53.76666*	*49.99539*	*52.23249*
Experiment 2				
0	*100*	*100*	*100*	*100*
1	*100*	*100*	*99.52381*	*99.52381*
2	*99.44444*	*99.44444*	*98.9709*	*98.9709*
3	*98.78148*	*98.78148*	*98.3111*	*98.3111*
4	*97.95831*	*97.95831*	*97.49184*	*97.49184*
5	*96.86988*	*96.86988*	*93.15887*	*96.40859*
6	*93.64088*	*95.25538*	*86.94827*	*94.80178*
7	*87.39816*	*92.0802*	*75.35517*	*88.48167*
Experiment 3				
0	*100*	*100*	*100*	*100*
1	*99.04762*	*99.04762*	*98.57143*	*99.52381*
2	*96.84656*	*96.84656*	*96.38095*	*98.41799*
3	*93.61834*	*89.09884*	*93.16825*	*95.79351*
4	*89.71758*	*79.44646*	*88.50984*	*92.6004*
5	*84.73327*	*67.08813*	*82.60918*	*88.48482*

Statistics	
Exp. 1	
Comparison of Survival Curves	
Log-rank (Mantel-Cox) Test	
Chi square	20.69
Df	3
P value	0.0001
P value summary	***

Are the survival curves sig different?	Yes
Log-rank test for trend	
Chi square	18.53
Df	1
P value	< 0.0001
P value summary	***
Sig. trend?	Yes
Exp. 2	
Comparison of Survival Curves	
Log-rank (Mantel-Cox) Test	
Chi square	6.211
Df	3
P value	0.1018
P value summary	ns
Are the survival curves sig different?	No
Log-rank test for trend	
Chi square	0.5263
Df	1
P value	0.4682
P value summary	ns
Sig. trend?	No
Exp. 3	
Comparison of Survival Curves	
Log-rank (Mantel-Cox) Test	
Chi square	34.01
Df	3
P value	< 0.0001
P value summary	***
Are the survival curves sig different?	Yes
Log-rank test for trend	
Chi square	5.312
Df	1
P value	0.0212
P value summary	*
Sig. trend?	Yes

Treatment: E. coli				
Av. number of mosquitoes per group: 30				
Experiment 1	*Percent Survival*			
Days	*dsCad*	*dsCaspar*	*dsCactus*	*dsGFP*
0	*100*	*100*	*100*	*100*
1	*99.52381*	*99.04762*	*99.52381*	*97.61905*
2	*97.31217*	*95.74603*	*98.41799*	*92.7381*
3	*94.71718*	*90.00127*	*95.13739*	*85.9373*
4	*90.77063*	*79.50112*	*87.20927*	*75.91129*
5	*85.72781*	*64.48425*	*76.55036*	*53.98136*
6	*77.15504*	*37.61581*	*54.86109*	*34.18819*
7	*61.72403*	*3.761581*	*21.94444*	*9.116852*
Experiment 2				
0	*100*	*100*	*100*	*100*
1	*97.85714*	*98.57143*	*100*	*100*
2	*94.59524*	*96.10714*	*98.33334*	*98.33334*
3	*90.81143*	*93.22393*	*93.41666*	*95.38333*
4	*86.27086*	*86.23213*	*85.24271*	*90.61417*
5	*77.64377*	*74.73451*	*72.4563*	*81.55275*
6	*64.05611*	*56.05089*	*45.28519*	*63.20338*
7	*12.81122*	*14.01272*	*9.057037*	*18.96101*
Experiment 3				
0	*100*	*100*	*100*	*100*
1	*97.14286*	*99.04762*	*99.04762*	*97.14286*
2	*91.20635*	*96.2963*	*94.6455*	*90.12698*
3	*81.47767*	*86.66666*	*84.54998*	*78.7109*
4	*63.1452*	*69.33334*	*68.34457*	*61.00095*
5	*42.79841*	*48.53333*	*48.60058*	*42.70066*
6	*22.11251*	*25.88445*	*24.30029*	*22.77369*
7	*0*	*0*	*0*	*0.7591229*

Statistics	
Exp. 1	
Comparison of Survival Curves	
Log-rank (Mantel-Cox) Test	
Chi square	50.45
df	3
P value	< 0.0001
P value summary	***
Are the survival curves sig different?	Yes
Log-rank test for trend	
Chi square	25.79
df	1
P value	< 0.0001
P value summary	***
Sig. trend?	Yes
Exp. 2	
Comparison of Survival Curves	
Log-rank (Mantel-Cox) Test	
Chi square	2.902

df	3
P value	0.407
P value summary	ns
Are the survival curves sig different?	No
Log-rank test for trend	
Chi square	0.1322
df	1
P value	0.7161
P value summary	ns
Sig. trend?	No

Exp. 3

Comparison of Survival Curves	
Log-rank (Mantel-Cox) Test	
Chi square	1.767
df	3
P value	0.6221
P value summary	ns
Are the survival curves sig different?	No
Log-rank test for trend	
Chi square	0.01741
df	1
P value	0.895
P value summary	ns
Sig. trend?	No

Treatment: S. aureus				
Av. number of mosquitoes per group: 30				
Experiment 1	*Percent Survival*			
Days	*dsCad*	*dsCaspar*	*dsCactus*	*dsGFP*
0	*100*	*100*	*100*	*100*
1	*99.52381*	*99.04762*	*99.52381*	*97.61905*
2	*97.31217*	*95.74603*	*98.41799*	*92.7381*
3	*94.71718*	*90.00127*	*95.13739*	*85.9373*
4	*90.77063*	*79.50112*	*87.20927*	*75.91129*
5	*85.72781*	*64.48425*	*76.55036*	*53.98136*
6	*77.15504*	*37.61581*	*54.86109*	*34.18819*
7	*61.72403*	*3.761581*	*21.94444*	*9.116852*
Experiment 2				
0	*100*	*100*	*100*	*100*
1	*98.09524*	*99.04762*	*100*	*94.7619*
2	*92.6455*	*96.2963*	*100*	*85.81217*
3	*86.46914*	*91.16049*	*99.33334*	*71.51014*
4	*78.5428*	*84.32346*	*96.02222*	*56.61219*
5	*61.08884*	*66.52184*	*91.75457*	*40.88659*
6	*40.72589*	*45.45659*	*85.6376*	*23.85051*
7	*10.86024*	*16.66742*	*74.21925*	*3.975085*
Experiment 3				
0	*100*	*100*	*100*	*100*
1	*96.66666*	*94.7619*	*99.04762*	*94.7619*
2	*91.2963*	*87.91799*	*97.39683*	*87.39153*
3	*84.60123*	*73.85111*	*82.46265*	*71.66106*
4	*76.84612*	*58.46546*	*66.6573*	*55.53732*
5	*67.45382*	*42.22506*	*49.62266*	*38.87613*
6	*53.96305*	*24.63128*	*30.60064*	*21.38187*
7	*32.37783*	*4.105214*	*7.14015*	*2.138187*

Statistics	
Exp. 1	
Comparison of Survival Curves	
Log-rank (Mantel-Cox) Test	
Chi square	50.45
df	3
P value	< 0.0001
P value summary	***
Are the survival curves sig different?	Yes
Log-rank test for trend	
Chi square	25.79
df	1
P value	< 0.0001
P value summary	***
Sig. trend?	Yes
Exp. 2	
Comparison of Survival Curves	
Log-rank (Mantel-Cox) Test	
Chi square	108

df	3
P value	< 0.0001
P value summary	***
Are the survival curves sig different?	Yes
Log-rank test for trend	
Chi square	6.216
df	1
P value	0.0127
P value summary	*
Sig. trend?	Yes

Exp. 3	
Comparison of Survival Curves	
Log-rank (Mantel-Cox) Test	
Chi square	34.89
df	3
P value	< 0.0001
P value summary	***
Are the survival curves sig different?	Yes
Log-rank test for trend	
Chi square	21.08
df	1
P value	< 0.0001
P value summary	***
Sig. trend?	Yes

Table S4. Kaplan-Meier survival analyses of *Cad* and *GFP* dsRNA-treated mosquitoes after sugar-feeding, blood-feeding, or *P. falciparum* infected blood-feeding.

Mosquitoes were either maintained on 10% sucrose solution only, provided a single naïve human blood meal and then maintained on 10% sucrose solution, or were provided with a *Pf*-infected blood meal (0.01-0.05% gametocytemia) and then maintained on a 10% sucrose solution. Three independent experiments were performed, and the data are provided below for each experiment.

Experiment 1	*Percent Survival*					
Days	*dsCad-Sucrose*	*dsGFP-Sucrose*	*dsCad-BF*	*dsGFP-BF*	*dsCad-PF*	*dsGFP-PF*
0	*100*	*100*	*100*	*100*	*100*	*100*
1	*100*	*100*	*100*	*100*	*100*	*100*
2	*99.68944*	*97.67081*	*98.49498*	*98.66221*	*99.49833*	*95.65218*
3	*97.52228*	*95.05754*	*96.71066*	*96.1599*	*98.77733*	*90.80025*
4	*94.51888*	*92.13004*	*92.8881*	*91.59896*	*95.65393*	*85.12524*
5	*89.47538*	*88.67062*	*87.83983*	*86.81989*	*90.24741*	*77.9874*
6	*82.27844*	*81.73118*	*79.14072*	*78.6412*	*83.70774*	*65.8372*
7	*74.32883*	*74.22928*	*70.10835*	*69.87955*	*74.83654*	*53.24225*
8	*66.24962*	*65.95917*	*60.74606*	*60.98184*	*65.54007*	*40.22105*
9	*58.01985*	*57.56064*	*51.282*	*51.92294*	*56.04151*	*27.10549*
10	*49.61118*	*49.00997*	*41.6945*	*42.66711*	*46.29516*	*13.84737*
11	*40.55174*	*39.84724*	*31.95067*	*32.46411*	*35.22458*	*0*
12	*31.29537*	*30.53511*	*21.99503*	*22.11323*	*23.99356*	
13	*21.77069*	*21.02055*	*11.71474*	*11.53734*	*12.51838*	
14	*11.12198*	*10.73876*	*0*	*0*	*0*	
15	*0*	*0*				

Experiment 1 Statistics	
Avg. number of mosquitoes per group: 46	
Comparison of Survival Curves	
Log-rank (Mantel-Cox) Test	
Chi square	*599.4*
df	*7*
P value	*< 0.0001*
P value summary	*****
Are the survival curves sig different?	*Yes*
Log-rank test for trend	
Chi square	*333.1*
df	*1*
P value	*< 0.0001*
P value summary	*****
Sig. trend?	*Yes*

Experiment 2	*Percent Survival*					
Days	*dsCad-Sucrose*	*dsGFP-Sucrose*	*dsCad-BF*	*dsGFP-BF*	*dsCad-PF*	*dsGFP-PF*
0	*100*	*100*	*100*	*100*	*100*	*100*
1	*99.5338*	*97.57576*	*99.6633*	*95.86777*	*99.0676*	*98.18182*
2	*98.27706*	*94.29037*	*99.13034*	*90.92912*	*97.06623*	*95.53719*
3	*96.65265*	*90.36161*	*98.1916*	*85.41827*	*94.65963*	*92.28024*
4	*94.30955*	*84.49397*	*97.19978*	*78.94719*	*91.79116*	*88.28542*
5	*90.18153*	*77.23944*	*96.14783*	*70.74488*	*88.08243*	*80.70536*
6	*84.37438*	*64.60026*	*93.6825*	*61.81245*	*81.74316*	*71.90114*
7	*78.16501*	*51.38657*	*91.08021*	*52.44693*	*75.0197*	*62.09644*
8	*67.50614*	*37.37205*	*87.06565*	*40.52717*	*65.9264*	*50.80618*
9	*55.64143*	*20.38476*	*82.58045*	*28.24621*	*56.3371*	*28.48225*
10	*42.57412*	*0*	*77.01948*	*14.97905*	*43.53322*	*0*
11	*28.81279*		*70.30945*	*0*	*30.34133*	
12	*14.84295*		*62.39583*		*16.0901*	
13	*0*		*53.57218*		*0*	
14			*44.48114*			
15			*34.70877*			
16			*23.48977*			
17			*12.10079*			
18			*0*			

Experiment 2 Statistics	
Avg. number of mosquitoes per group: 32	
Comparison of Survival Curves	
Log-rank (Mantel-Cox) Test	
Chi square	*517.4*
df	*7*
P value	*< 0.0001*
P value summary	*****
Are the survival curves sig different?	*Yes*
Log-rank test for trend	
Chi square	*63.52*
df	*1*
P value	*< 0.0001*
P value summary	*****
Sig. trend?	*Yes*

Experiment 3	*Percent Survival*					
Days	*dsCad-Sucrose*	*dsGFP-Sucrose*	*dsCad-BF*	*dsGFP-BF*	*dsCad-PF*	*dsGFP-PF*
0	*100*	*100*	*100*	*100*	*100*	*100*
1	*99.87302*	*99.67159*	*99.93651*	*99.9179*	*99.92982*	*99.68652*
2	*99.73985*	*99.24197*	*99.86988*	*99.83176*	*99.55972*	*98.65528*
3	*99.59987*	*98.52152*	*99.7998*	*99.65058*	*98.93503*	*96.76534*
4	*99.45231*	*97.6722*	*99.57803*	*99.17332*	*97.78078*	*92.80296*
5	*99.21831*	*96.68161*	*99.34372*	*98.67042*	*96.04246*	*87.31707*
6	*98.97026*	*95.63979*	*99.09536*	*97.18185*	*94.21308*	*80.54247*
7	*98.09052*	*93.88089*	*98.74302*	*94.38927*	*92.2805*	*72.21049*
8	*96.96949*	*91.56855*	*97.70857*	*91.36696*	*89.30702*	*59.76041*
9	*95.77602*	*89.13968*	*96.60622*	*88.21637*	*86.16774*	*46.02238*
10	*94.49901*	*86.57819*	*95.42548*	*84.92094*	*82.83592*	*26.18515*
11	*92.43721*	*83.86414*	*94.15314*	*80.92779*	*78.66344*	*0*
12	*88.86297*	*80.24931*	*92.27007*	*75.62562*	*73.41922*	
13	*85.04515*	*76.40595*	*90.21963*	*70.12031*	*66.28706*	
14	*80.93464*	*72.28925*	*87.96413*	*64.37769*	*56.12305*	
15	*76.3098*	*66.05742*	*84.61312*	*57.71793*	*45.49709*	
16	*69.52671*	*58.08497*	*78.0321*	*50.25441*	*34.42613*	
17	*58.40244*	*49.67266*	*64.50654*	*42.10973*	*23.25676*	
18	*46.72195*	*40.46609*	*50.3151*	*33.57888*	*11.78343*	
19	*32.39388*	*28.60534*	*34.66151*	*24.31574*	*0*	
20	*16.4129*	*15.04246*	*17.56183*	*13.83482*		
21	*0*	*0*	*0*	*0*		

Experiment 3 Statistics	
Avg. number of mosquitoes per group: 66	
Comparison of Survival Curves	
Log-rank (Mantel-Cox) Test	
Chi square	***2606***
df	***7***
P value	***< 0.0001***
P value summary	*****
Are the survival curves sig different?	***Yes***
Log-rank test for trend	
Chi square	***74.53***
df	***1***
P value	***< 0.0001***
P value summary	*****
Sig. trend?	***Yes***

Table S5. Statistical analyses of fecundity and fertility in *Cad* and *GFP* dsRNA-treated and uninjected mosquitoes.

The fecundity and larval hatch-rate assays were performed for three biological replicates, and the number of eggs laid by each female and their hatch rate were used to calculate the mean value. Statistical significance was determined using the Mann-Whitney test. As a note, fecundity values and larval hatch-rates for uninjected controls were quite similar to a previous study using the same strain of *An. gambiae* [31].

Fig. 4B. Fecundity	***dsGFP***	***dsCad***	***Uninjected***
n=	44	46	90
Median (with zeros)	24	9	30
Mean (with zeros)	23.5	16	29.3
Mann-Whitney test *p*-value	**0.0301 (dsCad compared to dsGFP); **0.0004 (dsCad compared to uninjected)*		
Fig. 4C. Fertility	***dsGFP***	***dsCad***	***Uninjected***
n=	44	46	90
Mean (with zeros)	45.1%	29.1%	41.6%
Mann-Whitney test *p*-value	**0.0317 (dsCad compared to dsGFP); *0.0261 (dsCad compared to uninjected)*		

Table S6. Bacteria species isolated from the midguts of *Cad* dsRNA-treated *An. gambiae* mosquitoes.

Bacterial genus	Closest Match (NCBI Accession #)	Max. Identity[a]
Asaia	*Asaia sp.* AA 9.6 (FN814295.1)	99%
Gamma proteobacterium	*Gamma proteobacterium* A-4 (FJ871128.1)	97%
Rahnella	*Rahnella sp.* CONC2 (EU275360.1)	99%

[a]Maximum identity to the most closely related sequence determined via the BLASTn program.

Table S7. Bacteria species isolated from the midguts of *GFP* dsRNA-treated *An. gambiae* mosquitoes.

Bacterial genus	Closest Match (NCBI Accession #)	Max. Identity[a]
Acinetobacter	*Acinetobacter calcoaceticus* strain NBRAJG93 (EU661709.1)	99%
Asaia	*Asaia krungthepensis* strain G3-3-08 (FJ816021.1)	99%
Enterobacter	*Enterobacter sp.* ICB551 (HM748088.1)	99%
Pantoea	*Pantoea agglomerans* strain AR_PINLBH4 (HM582878.1)	99%
Pseudomonas	*Pseudomonas sp.* JCM 17186 (AB602346.1)	99%
Serratia	*Serratia marcescens* strain C3 (GU212864.1)	100%
Rahnella	*Rahnella sp.* CONC2 (EU275360.1)	99%

[a]Maximum identity to the most closely related sequence determined via the BLASTn program.

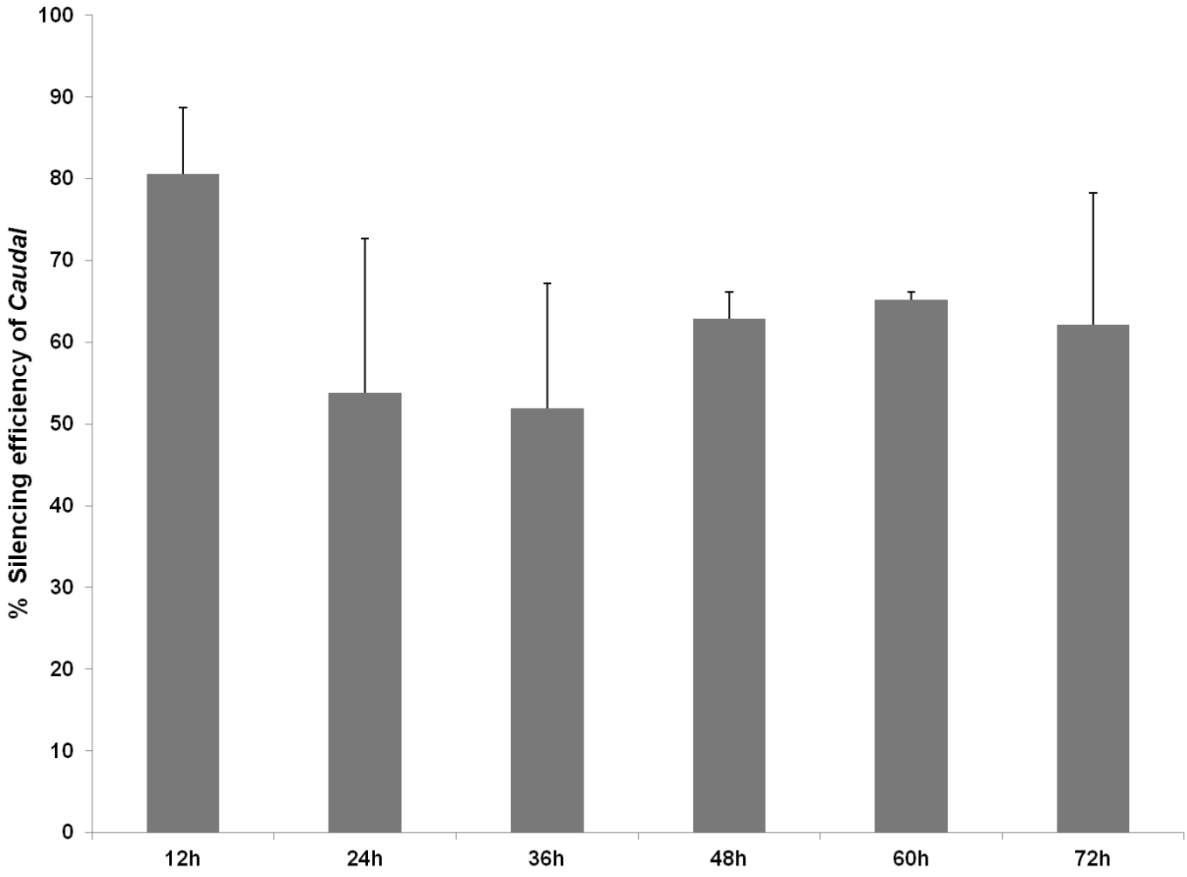


Figure S1. Silencing efficiency of *Cad*. *Cad* silencing efficiency in *Cad* dsRNA-treated mosquito midguts compared to *GFP* dsRNA-treated mosquito midguts. Columns represent the average percent silencing efficiency of *Cad* for three biological replicates. Error bars represent the standard error of the mean. The ribosomal gene *S7* was used as an internal control.

APPENDIX B: SUPPLEMENTARY DATA

Table S1. Selected bacterial isolates derived from the midguts of laboratory *An. gambiae* mosquitoes.

Bacterial genus	Designated Name	Closest Match (NCBI Accession #)	Max. Identity[a]
Acinetobacter	*Aci_sp_Ag*	*Acinetobacter calcoaceticus* strain NBRAJG93 (EU661709.1)	99%
Pseudomonas	*Pse_sp_Ag*	*Pseudomonas sp.* JCM 17186 (AB602346.1)	99%

[a]Maximum identity to the most closely related sequence determined via the BLASTn program.

Table S2. Selected bacterial isolates derived from the midguts of laboratory *Ae. aegypti* mosquitoes.

Bacterial genus	Designated Name	Closest Match (NCBI Accession #)	Max. Identity[a]
Escherichia	*Esc_sp_Ae*	*Escherichia fergusonii* strain i_10_chl (JQ838153.1)	99%
Pseudomonas	*Pse_sp_Ae*	*Pseudomonas rhodesiae* strain CIP 104664T (AB021410.1)	99%
Asaia	*Asa_sp_Ae*	*Asaia sp.* strain AA 9.6 Ab (FN814295.1)	99%

[a]Maximum identity to the most closely related sequence determined via the BLASTn program.

Table S3. Primers used for gene expression analysis, dsRNA synthesis, qRT-PCR assessment of bacterial load, and qRT-PCR validation of RNAi-mediated gene

silencing and the efficiencies of gene silencing. Underlined letters indicate the T7 promoter sequence of the forward (RNAiF) and reverse RNAi primers (RNAiR) used for dsRNA synthesis. For gene silencing validation, KD% (± SEM) denoting the mean percent efficiency of gene knock-down (KD %) with standard error of the mean (SEM) can be found in previous studies (for example, [41,200]). Specifically, the silencing efficiency will be determined at 2-3 d post dsRNA injection. It is important to note that the silencing efficiency will be assessed in the midguts of dsRNA-injected mosquitoes. Abbreviations: Ag-*Anopheles gambiae*; Ae-*Aedes aegypti*; Cad-*Caudal*.

Gene Name	Primer Name	Primer sequence (5'-3')
AgS7	AgS7-F	CCATCCTGGAGGATCTGGTA
	AgS7-R	GATGGTGGTCTGCTGGTTCT
AeS7	AeS7-F	GGGACAAATCGGCCAGGCTATC
	AeS7-R	TCGTGGACGCTTCTGCTTGTTG
AgCad	AgCad-F	ACCAGAGTCAACCCAATCCA
	AgCad-R	ATCGTGATGTAGCGCGTGTA
AeCad	AeCad-F	ACAGTCTGAATATTTCATTCCATCC
	AeCad-R	AACGGTATTCATGGGGTTCA
AgIMD	AgIMD-F	CGAAGCTAGAGACCGATGCT
	AgIMD-R	ATTCCCATTTTGCGTAGCAG
AeIMD	AeIMD-F	TCATTCCGCGAAGGGCTGGC
	AeIMD-R	AGCGCAGAAACATCGTTCGCA
16S rRNA universal	16s-F	TCCTACGGGAGGCAGCAGT
	16s-R	GGACTACCAGGGTATCTAATCCTGTT
16S rRNA Pse_sp_Ag	16s-F	AAGCAACGCGAAGAACCTTA
	16s-R	CACCGGCAGTCTCCTTAGAG
16S rRNA Aci_sp_Ag	16s-F	CAGCTCGTGTCGTGAGATGT
	16s-R	CGTAAGGGCCATGATGACTT
16S rRNA Esc_sp_Ae	16s-F	CAGCCACACTGGAACTGAGA
	16s-R	GTTAGCCGGTGCTTCTTCTG
16S rRNA Asa_sp_Ae	16s-F	CAGCTCGTGTCGTGAGATGT
	16s-R	CACTGTCACCGCCATTGTAG
16S rRNA Pse_sp_Ae		
	16s-F	AAGCAACGCGAAGAACCTTA
	16s-R	CACCGGCAGTCTCCTTAGAG
GFP	GFP-RNAiF	TAATACGACTCACTATAGGATGGTGAG CAAGGGCGAGGAGCTGT

	GFP-RNAiR	TAATACGACTCACTATAGGTTACTTGTACAGCTCGTCCATGCCG
AgCad	AgCad-T7F	TAATACGACTCACTATAGGGCCATGTACTATCCCCATCCG
	AgCad-T7R	TAATACGACTCACTATAGGGTGTCTTTCGTGCGTGTCTTC
AeCad	AeCad-T7F	TAATACGACTCACTATAGGGACACCC
	AeCad-T7R	TAATACGACTCACTATAGGGTCGAAC
AgIMD	AgIMD-T7F	TAATACGACTCACTATAGGAATTTCCCAAATGGTGTG
	AgIMD-T7R	TAATACGACTCACTATAGTGTGTAGATTGCTCGCGTTC
AeIMD	AeIMD-T7F	TAATACGACTCACTATAGGACCGAAGAAGACCGCACAAGGC
	AeIMD-T7R	TAATACGACTCACTATAGGGTGCCGAGCGTTGGTTCGTCG

Table S4. *In-vitro* growth inhibition activity of *Anopheles*-derived bacteria isolates.

Anopheles-derived bacterial isolates *Zone of Inhibition Diameter in mm (± SEM)*		
	Pse_sp_Ag	*Aci_sp_Ag*
Pse_sp_Ag	0 ± 0 mm	0 ± 0 mm
Aci_sp_Ag	0 ± 0 mm	0 ± 0 mm

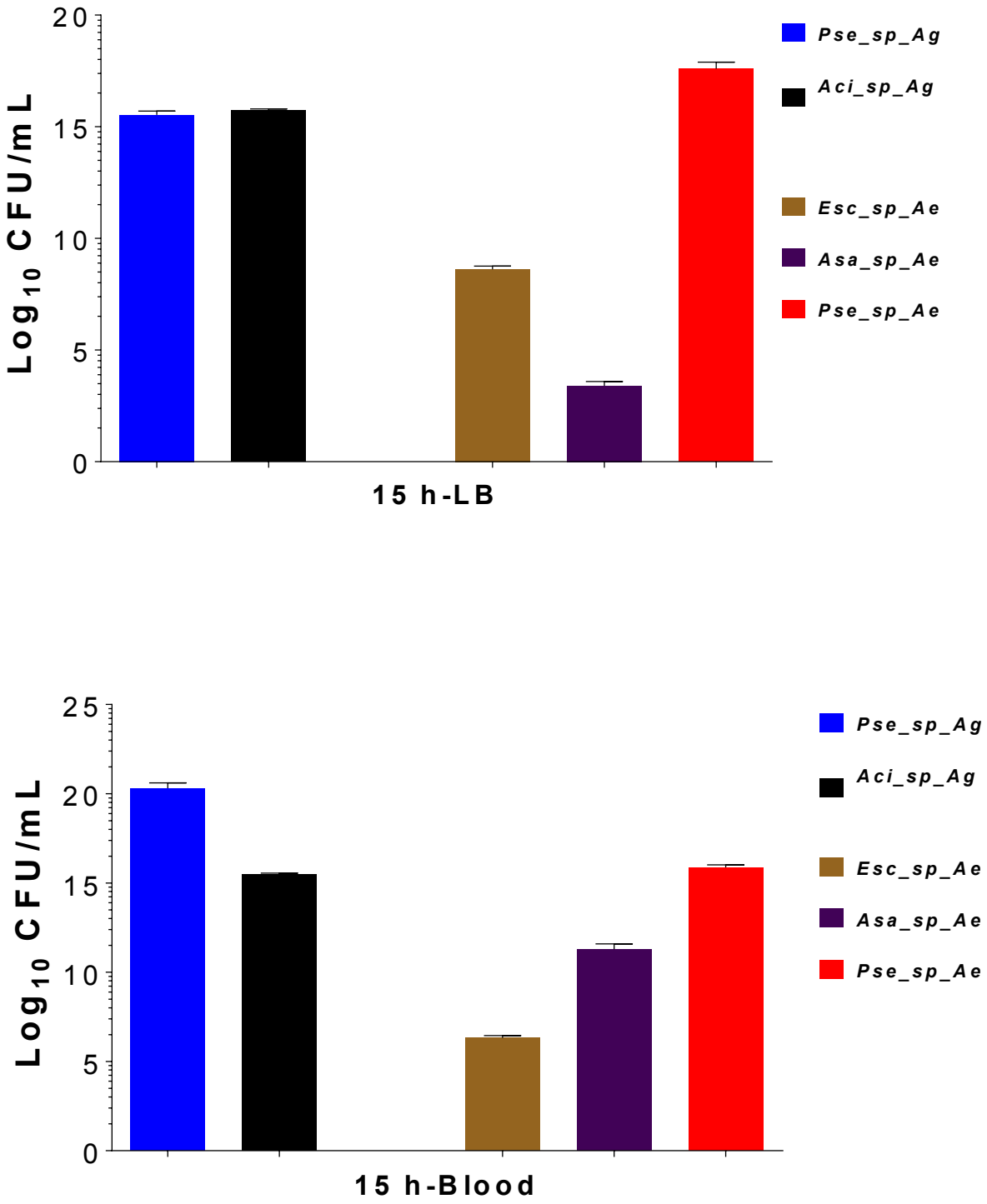
Table S5. *In-vitro* growth inhibition activity of *Aedes*-derived bacteria isolates.

Aedes-derived bacterial isolates *Zone of Inhibition Diameter in mm (± SEM)*

	Esc_sp_Ae	*Asa_sp_Ae*	*Pse_sp_Ae*
Esc_sp_Ae	0 ± 0 mm	0 ± 0 mm	0 ± 0 mm
Asa_sp_Ae	0 ± 0 mm	0 ± 0 mm	0 ± 0 mm
Pse_sp_Ae	0 ± 0 mm	0 ± 0 mm	0 ± 0 mm

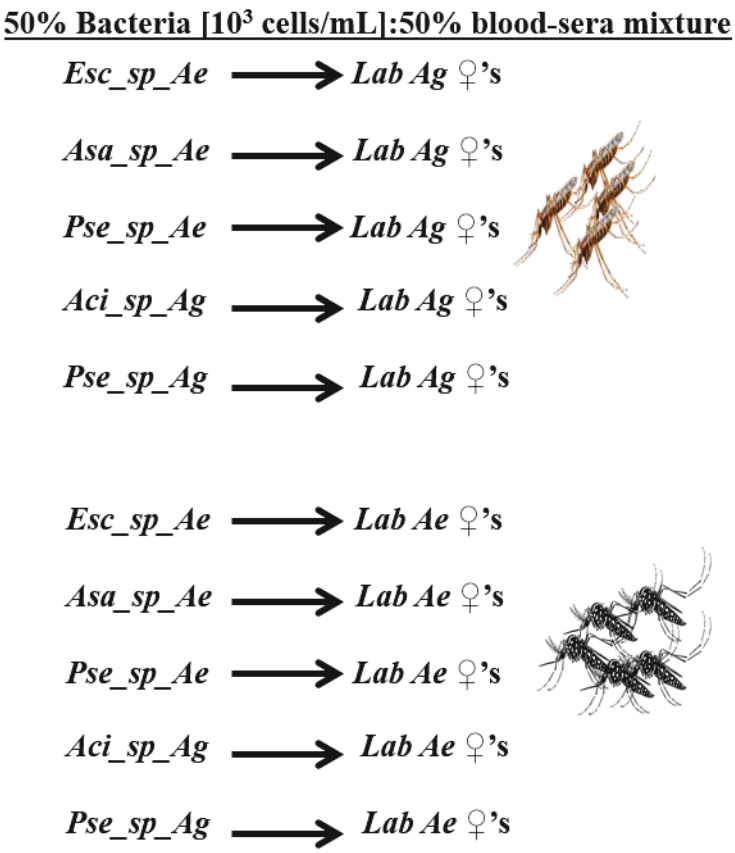
Table S6. *In-vitro* antibiotic resistance activity of bacteria isolates.

Zone of Inhibition Diameter in mm (± SEM)				
Bacterial isolates	10,000 ug/mL PS	100 ug/mL PS	10 ug/mL PS	1 ug/mL PS
Pse_sp_Ag	27 ± 1 mm	0 ± 0 mm	0 ± 0 mm	0 ± 0 mm
Aci_sp_Ag	32 ± 1 mm	14 ± 2 mm	0 ± 0 mm	0 ± 0 mm
Esc_sp_Ae	0 ± 0 mm	0 ± 0 mm	0 ± 0 mm	0 ± 0 mm
Asa_sp_Ae	32 ± 1 mm	22± 1 mm	0 ± 0 mm	0 ± 0 mm
Pse_sp_Ae	29 ± 1 mm	0 ± 0 mm	0 ± 0 mm	0 ± 0 mm

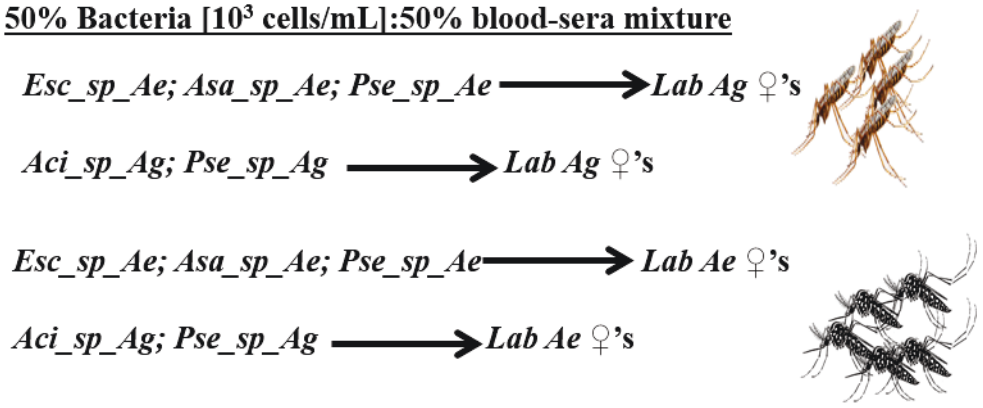


Supplementary Figure 1. *In-vitro* growth dynamics of bacterial isolates. To assess if

our bacterial isolates can grow in blood, we inoculated 200 ul of OD_{600} .5 bacterial isolate in 2 mL 50% blood:50% LB liquid media and grew overnight for 15 h. As controls, we grew each bacterial isolate (OD_{600} .5) in 2 mL of LB liquid media for the same amount of time. We conducted CFU assays. One-way ANOVA with a Tukey's multiple comparison test was conducted to see if the presence of blood significantly influenced growth. No statistical significance was detected. CFU/mL is expressed on a $\log_{10}$ scale (y-axis). Error bars represent the standard error of the mean. Three replicates were conducted.



Supplementary Figure 2. Individual *Anopheles* and *Aedes*-derived bacterial isolates feeding scheme.



Supplementary Figure 3. Grouped *Anopheles* or *Aedes*-derived bacterial isolates feeding scheme.

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CURRICULUM VITAE

April Melinda Clayton
Ph. D. Candidate
Johns Hopkins School of Medicine
Graduate Program in Biochemistry, Cellular, and Molecular Biology (BCMB)

Address: 1 East Chase St.
Apt. 603
Baltimore, MD 21202
Tel. 864-910-2787
E-mail: aclayton@jhmi.edu; april.m.clayton@gmail.com

Educational History:

- **PhD expected:** September 2013 The Johns Hopkins School of Medicine's Biochemistry, Cellular, and Molecular Biology (BCMB) Graduate Program. Ph.D. Thesis Advisor: Dr. George Dimopoulos, Johns Hopkins Bloomberg School of Public Health, Department of Microbiology and Molecular Immunology.
- **BS:** May 2008 Erskine College, B.S. Biology and Chemistry, Departmental honors in biology and chemistry, Summa cum laude.
- **HS:** May 2004 SC Governor's School for Science and Mathematics.
- **Certificates:**
  - July 2013: Johns Hopkins School of Medicine Institute for Excellence in Education Summer Teaching Camp.
  - May 2013: National Institutes of Health Office of Intramural Training and Education's Scientists Teaching Science Online Pedagogy Course.
  - August 2009: Graduate of the Woods Hole Marine Biological Laboratory's Biology of Parasitism Modern Approaches Course.

Awards/Fellowships in Science (High School-Graduate):

- *UNCF/Merck Science Initiative (UMSI) Graduate Science Research Dissertation Fellow (September 2012-September 2013).*
- *The National Science Foundation Graduate Research Fellowship Program Recipient (September 2009-September 2012).*
- 63rd Lindau Nobel Laureate Meeting Young Researcher Participant (June 30-July 6, 2013, Lindau, Germany).
- Bayer-Lindau Fellowship (Summer 2013).
- American Society for Microbiology Student Travel Award for the ASM 2012 meeting (June 16-19, 2012, San Francisco, California).
- Ford Foundation Fellowship 2012 Dissertation Competition Awardee (April 2012).
- Philanthropic Educational Organization (PEO) Scholar (April 2012-September 2013).
- Intramural NIAID Research Opportunities (INRO) Scholar and Fellow (February 2012-Current).

- Carl Storm Underrepresented Minority Fellowship (CSURM) for the 2011 Tropical Infectious Diseases Gordon Research Conference (Fall 2010).
- The JHMI BCMB Graduate Program Student Colloquium. Presented Oral Presentation-Second Place Award (April 2010).
- Johns Hopkins School of Medicine Annual Graduate Student Association Poster Session. Presented Poster- First Place, The Junghea Park Award (April 2010).
- Keystone Symposia Scholarship (National Institute of Allergy and Infectious Diseases (NIAID) Scholarship) for the meeting, Molecular Targets for Control of Vector-Borne Diseases: Bridging Lab and Field Research (Apr 11 - Apr 16, 2010, Copper Mountain, Colorado).
- American Society for Cell Biology Minority Scholarship (Summer 2009).
- William Townsend Porter Scholarship (Summer 2009).
- The Johns Hopkins School of Medicine's Turock Young Scientist Award (Summer 2009).
- Department of Health and Human Services Public Health Service's Ruth L. Kirschstein National Research Service Award/Graduate Student Fellowship (July 2008-September 2009).
- Erskine College's Ellison Biology Award (2008).
- 2nd Place for the Frank G. Brooks Award for Excellence in Student Research at the South East Region District I Beta Beta Beta Convention (2008).
- American Chemical Society's 2008 Outstanding Senior Chemistry Major at Erskine College (2008).
- The President's Award for Undergraduate Research Presentation at the Southeastern Branch of the American Society of Microbiology Meeting (Fall 2007).
- The Johns Hopkins Summer Internship Program Scholar (Summer 2007).
- Leadership Alliance Scholar (Summer 2007).
- Dr. & Mrs. James Boyce Memorial Scholarship in Biology, Erskine College (2007).
- SC Academy of Science Invertebrate Biology Research Award (Spring 2007).
- SC Academy of Science Award for Outstanding Undergraduate Research in Cell Biology (Spring 2007).
- Honorable Mention for the Barry M. Goldwater Scholarship (2007).
- Honorable Mention for the President's Award for Undergraduate Research Presentation at the Southeastern Branch of the American Society of Microbiology Meeting (Fall 2006).
- Dr. Calvin G. Reid Biology Award and Scholarship, Erskine College (2006).
- The Due West Branch of the American Association of University Women Award for Biology and Chemistry, Erskine College (2005).
- Dr. and Mrs. E. L. Reid Chemistry Scholarship, Erskine College (2005-2007).
- 5th Place in Best Oral Research Presentation at SC Junior Academy of Science (Spring 2003).

Academic Accomplishments (Undergraduate-Graduate):

- Ph. D Candidacy in the Johns Hopkins School of Medicine's Biochemistry, Cellular, and Molecular Biology Graduate Program (March 25, 2010).
- Erskine College's H.M. Young Award (2008).
- ODK (Omicron Delta Kappa) Leader of the Year for the Erskine College Circle (Spring 2008).
- J. Lacy McLean Scholarship-SC Independent College Student of the Year Award (2007-2008).
- Erskine College Presidential Scholar (Fall 2004-Spring 2008).
- Erskine College's Garnet Academic Circle (Fall 2004-Spring 2005).
- Erskine College's Dean's List (Fall 2004, Spring 2005, Spring 2006, Fall 2006, Spring 2007, Fall 2007, and Spring 2008).

High School & Undergraduate Summer Research Experiences:

- The Johns Hopkins University Summer Internship Program from May 2007 to August 2007.
- Biology Externship at the Greenwood Genetic Center in January 2007.
- Clemson University REU in Genetics and Biochemistry from May 2006 to July 2006.
- Summer Research at Erskine College from May 2005 to August 2005.
- S.C. Governor's School for Science and Mathematics' Summer Research Program at Clemson University from May 2003 to July 2003.

Memberships in Science & Academia (Undergraduate-Graduate):

- International Black Doctoral Network (Summer 2013-Current).
- Student Member of the Society for Advancement of Chicanos and Native Americans in Science (Winter 2009-Current).
- Student Member of the American Society for Microbiology (Winter 2009-Current).
- Student Member of the American Society of Tropical Medicine and Hygiene (Fall 2009-Current).
- Johns Hopkins School of Medicine's Biomedical Scholars Association Member (Fall 2008-Current).
- American Association of University Women Member (Spring 2008-Current).
- Student Member of the American Chemical Society (Fall 2006-Current).
- Regular Member of the Association of Southeastern Biologists (Summer 2006-Current).
- Student Member of the Southeastern Branch of the American Society of Microbiology (Fall 2005-2009).
- Omicron Delta Kappa, The National Leadership Honor Society, Member (Fall 2007-Current).
- Beta Beta Beta: Biological Honors Society, Erskine College Chapter (Associate Member: Fall 2004-Spring 2006, Full Member: Fall 2006-Spring 2008).

Professional/Scholarly Service and Activities (Undergraduate-Graduate):

- Johns Hopkins School of Medicine's Biomedical Scholars Association (Secretary: Fall 2009-Spring 2010) (President: Summer 2010-Summer 2011).
- The Johns Hopkins School of Medicine's Biochemistry, Cellular, and Molecular Biology Graduate Program's Student Seminar Committee (Steering Committee Member: Fall 2009-Fall 2010).
- The Baltimore Albert Schweitzer Fellowship Program (Fellow: March 2009-March 2010; Fellow for Life: March 2010-Current).
- Biomedical Scholars Association's Junior Biomedical Scholars Research Program (Research Mentor Fall 2008- Spring 2011).
- Incentive Mentoring Program (Mentor for High School Student Miss Kierra Alsup: Fall 2008-Fall 2009) (Co-director of Community Service: Feb. 2009-March 2010) (Director of Community Service: March 2010-Sep. 2010) (Head of Household Mentor for High School Student Miss Diamond Coles: March 2010-March 2011).
- Omicron Delta Kappa: The National Leadership Honor Society (Fall 2007-Current).
- The Erskine College Strategic Planning Academic Committee (Fall 2007-Spring 2008).
- The Erskine Green Committee (Fall 2007-Spring 2008).
- Class of 2008 Secretary and Treasurer, Erskine College (Fall 2004-Spring 2008).
- Beta Beta Beta (Fall 2004-Spring 2008) (Secretary: Spring 2007-Spring 2008).
- Phi Lambda Sigma Literary Society (Spring 2005-Spring 2008) (Secretary: Spring 2006-2007) (President: Spring 2007-Spring 2008).
- Association of Multicultural Students (Fall 2004-Spring 2008) (Vice-President: Spring 2006-Spring 2007) (President: Spring 2007-Spring 2008).
- Erskine College Ambassador (Fall 2004-Spring 2008).

Scientific Meetings and Presentations (Graduate):

- **July 26, 2013.** The Johns Hopkins Malaria Research Institute Malaria Friday Seminar. **Oral Presentation: *Caudal* is a negative regulator of the *Anopheles* IMD pathway that controls resistance to P*lasmodium falciparum* infection.** Authors: **April Clayton**, Chris Cirimotich, Yuemei Dong, and George Dimopoulos.
- **July 2, 2013.** Bayer Lindau Science Dialogue &Poster Session. **Presented Poster: *Caudal* is a negative regulator of the *Anopheles* IMD pathway that controls resistance to P*lasmodium falciparum* infection.** Authors: **April Clayton**, Chris Cirimotich, Yuemei Dong, and George Dimopoulos.
- **June 16-19, 2012.** The 112th American Society for Microbiology. **Young Investigator Oral Presentation: The Innate Immune Gene *Caudal* is a *Plasmodium falciparum* Agonist in *Anopheles gambiae.*** Authors: **April Clayton**, Chris Cirimotich, Yuemei Dong, and George Dimopoulos.
- **December 4-8, 2011.** The American Society of Tropical Medicine and Hygiene's 60th Annual Meeting. **Oral Presentation: *Caudal, a Plasmodium falciparum agonist***. Authors: **April Clayton**, Suchismita Das, Jayme Souza-Neto, Musapa Mulenga, Chris Cirimotich, and George Dimopoulos.

- **July 29, 2011.** The Johns Hopkins Malaria Research Institute Malaria Friday Seminar. **Oral Presentation: *Caudal*, a *Plasmodium falciparum* agonist**. Authors: **April Clayton** & George Dimopoulos.
- **April 11, 2011.** The JHMI BCMB Graduate Program Student Colloquium. **Oral Presentation: *Caudal*'s role in the tripartite interactions between the mosquito *Anopheles* innate immune system, the microbiota, and the malaria parasite *Plasmodium.*** Authors: **April Clayton** & George Dimopoulos.
- **November 3-7, 2010.** The American Society of Tropical Medicine and Hygiene's 59th Annual Meeting. **Presented Poster: *Caudal* controls vector competence for *Plasmodium falciparum* as a regulator of the tripartite interactions between the innate immune system, the microbiota, and the malaria parasite.** Authors: **April Clayton,** Suchismita Das, Musapa Mulenga, Jayme Souza-Neto, Chris Cirimotich, and George Dimopoulos.
- **June 7-8, 2010.** Johns Hopkins Malaria Research Institute Research Advances in Malaria: Biology of Mosquito Vectors Conference. **Presented Poster: *Caudal* modulates Anopheline anti-*Plasmodium* defense and midgut microbiota.** Authors: **April Clayton** & George Dimopoulos.
- **April 27, 2010.** The JHMI BCMB Graduate Program Student Colloquium. **Oral Presentation: The Mosquito *Anopheles*' Innate Immunity and the Midgut Microflora battle the malaria parasite *Plasmodium.*** Authors: **April Clayton** & George Dimopoulos. **Second Place Award.**
- **April 11-16, 2010.** Keystone Symposia for Molecular Targets for Control of Vector-Borne Diseases: Bridging Lab and Field Research. **Presented Poster: *Caudal* modulates the *Anopheles gambiae* anti-*Plasmodium* defense response.** Authors: **April Clayton** & George Dimopoulos.
- **April 7, 2010.** Johns Hopkins School of Medicine Annual Graduate Student Association Poster Session. **Presented Poster: *Caudal's* role on the Anopheline microbiota and its influence on *Plasmodium* infection.** Authors: **April Clayton** & George Dimopoulos**. First Place Award: The Junghea Park Award.**
- **November 18-22, 2009.** The American Society of Tropical Medicine and Hygiene's 58th Annual Meeting. **Presented Poster: *Caudal* and *Peptidoglycan Recognition Protein LA* (*PGRPLA*) in *Anopheles gambiae* anti-*Plasmodium* defense.** Authors: **April Clayton** & George Dimopoulos.
- **April 26-29, 2009.** The Woods Hole Marine Biological Laboratory's Kinetoplastid Molecular Cell Biology Meeting. **Presented Poster: Knockout of *T. brucei* acyl carrier protein causes kDNA loss.** Authors: **April Clayton***, Jennifer Guler*, Megan Lindsay*, Eva Gluenz, Keith Gull, Terry Smith, and Paul Englund. *These authors contributed equally to this work.

Internships (Graduate):

- Scientific Writer for the AIDS Beacon (March 2011-October 2011).

Teaching Experiences (Graduate):

- Lecturer for Life on the Edge (BL 107) at Loyola University Maryland (January

2013-Current).
- Lecturer for General Genetics (BL 281) at Loyola University Maryland (January 2013-May 2013).
- Lab Instructor for Organismal Biology (BL 126) at Loyola University Maryland (January 2012-May 2012).
- Lab Instructor for Cellular and Molecular Biology (BL 119) at Loyola University Maryland (September 2011-December 2012).

Teaching Experiences (Undergraduate):
- Assistant for the Summer Science Program at the SC Governor's School for Science and Mathematics (Ecology and Forensic Science) (Summer 2008).
- Lab assistant for Advanced Biochemistry at Erskine College (Spring 2008).
- Lab assistant for General Chemistry 101 & 102 at Erskine College (Fall 2005-Fall 2007).
- Assistant for the Summer Science Program at the SC Governor's School for Science and Mathematics (Entomology and Marine Biology) (Summer 2004).

Scientific Mentoring Experiences (Graduate):
- Johns Hopkins Malaria Research Institute (JHMRI) Summer Internship Program (Summer 2012).
    - Research Mentor to undergraduate student Mr. Nathan Fastman (University of Maryland Baltimore County).
- Research Mentor to master's student Ms. Alma Ortiz (Instituto Conmemorativo Gorgas de Estudios de la Salud) (Summer 2011 & 2012).
- Biomedical Scholars Association's Junior Biomedical Scholars Research Program (Fall 2008- Spring 2011).
    - Research Mentor to Dunbar High School students.

International Research Experiences (Graduate):
- Field Research in Puerto Rico (November 2011).
    - *Microbiome analyses of field Culex, Anopheles, and Aedes mosquitoes.*
- Field/Lab Research at the Malaria Institute in Macha (MIAM), Zambia (Feb-March 2010).
    - *Analyzing the microbiome and innate immune defense system of field Anopheles arabiensis.*

Publications (Graduate):
- **Clayton, A.M.**, Fastman, N., and Dimopoulos, G. **(2013)**. Cross-colonization and co-adaptation capacities between the midgut microbiota and the phylogenetically distinct mosquito species *Anopheles gambiae* and *Aedes aegypti*. **In preparation.**
- **Clayton, A.M.**, Dong, Y., and Dimopoulos, G. **(2013)**. Scientific Review: *Anopheles* innate immune defenses against *Plasmodium* infection. **Submitted and Accepted to the Journal of Innate Immunity**.

- **Clayton, A.M.**, Cirimotich, C., Dong, Y., and George Dimopoulos, G. **(2013)**. *Caudal* is a negative regulator of the *Anopheles* IMD Pathway that controls resistance to *P. falciparum* infection. Dev Comp Immunol. 2013 Apr;39(4):323-32. doi: 10.1016/j.dci.2012.10.009. Epub 2012 Nov 22.
- Cirimotich, C.M., **Clayton, A.M.**, and Dimopoulos, G. **(2011)**. Low- and high-tech approaches to control *Plasmodium* parasite transmission by *Anopheles* mosquitoes. J Trop Med. 2011;2011:891342. Epub 2011 Aug 17.
- Cirimotich, C.M., Dong, Y., **Clayton, A.M.**, Sandiford, S., Souza-Neto, J., Mulenga, M., and Dimopoulos, G. **(2011)**. Natural microbe-mediated refractoriness to *Plasmodium* infection in *Anopheles gambiae.* Science. 2011 May 13;332(6031):855-8.
- **Clayton, A.M.***, Guler, J.L.*, Lindsay, M.E.*, Gluenz, E., Gull, K., Smith, T.K., Jensen, R.E., and Englund, P.T. **(2011)**. Depletion of mitochondrial acyl carrier protein in bloodstream form *Trypanosoma brucei* causes a kinetoplast segregation defect. Eukaryotic Cell. 2011 Mar;10(3):286-92. Epub 2011 Jan 14.